FULL REPORT

Chemical Constituents of *Cassia alata* Linn., Antibacterial, Anticancer, and Antioxidation Activities

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EXECUTIVE SUMMARY

The diversities of plants in Thailand are found to possess several medicinal properties. Medicinal plants have been widely used in the treatment of illness and diseases for centuries. Pure compounds extracted from many plants and many parts of the plants are explored and tested for biological activities. Plants of the family Leguminosae is the world's most important species because a large number of these families are used in Thai traditional medicine. They have been found to be a source of different secondary metabolites, flavonoids, anthraquinones, and xanthones with various biological activities. *Cassia alata* Linn. is one of a species in *Cassia* genus, family of Leguminosae. Therefore, *C. alata* was chosen for the phytochemical investigation as well as the evaluation of antibacterial, anticancer, and antioxidation activities of the crude extracts and the isolated compounds.

This research involved the phytochemical investigation of flowers, leaves, roots, stems, and twigs of *Cassia alata* Linn. by chromatographic techniques and crystallization. All isolated pure compounds were characterization by UV, IR, and NMR spectroscopic methods. Antibacterial activity against three Gram-positive bacteria (*B. cereus*, *S. aureus*, MRSA SK1) and three Gram-negative bacteria (*E. coli*, *P. aurenginosa*, and *S. typhimurium*) were evaluated by broth microdilution method. Anticancer and antioxidation activities of the crude extracts and isolated pure compounds were evaluated using resazurin microplate assay and DPPH assay, respectively.

Phytochemical investigation of the extracts of *C. alata* Linn. yielded 23 compounds: hydroxyquinol (**1**), 2′,6′-dihydroxy-4′-methoxydihydrochalcone (**2**), stigmasterol (**3**), ziganein (**4**), aloe-emodin (**5**), emodin (**6**), kaempferol (**7**), diosmetin (**8**), physcion (**9**), *β*-sitosterol (**10**), lupeol (**11**), caffeic acid (**12**), apigenin (**13**), *trans*-resveratrol (**14**), ω-hydroxyemodin (**15**), orientalone (**16**), euxanthone (**17**), 3-geranyloxy-1,7-dihydroxyxanthone (**18**), *trans*-dihydrokaempferol (**19**), luteolin (**20**), lunatin (**21**), 7,4′-dihydroxy-5-methoxyflavone (**22**), and hydroquinone (**23**). Moreover, Sixteen compounds (**1**, **2**, **4**, **8**, **9**, **11-19**, **21**, and **22**) were reported for the first time instance as constituents of *C. alata* Linn.

The crude extracts of *C. alata* showed moderate antibacterial activity against gram-positive (*Bacillus cereus* TISTR 687, *Staphylococcus aureus* TISTR 1466 and methicillin resistant *Staphylococcus aureus* (MRSA)-SK1) and weak antibacterial activity against gram-negative (*Escherichia coli* TISTR 780, *Pseudomonas aeruginosa* TISTR 781 and *Salmonella typhimurium* TISTR 292). Compounds **2** and **6** exhibited strong antibacterial activity against *Bacillus cereus* TISTR 687 and methicillin resistant *Staphylococcus aureus* SK1with MICs values of 8 and 4 μg/mL, respectively. Whereas, the dichloromethane and acetone extracts of *C. alata* stems showed inactive anticancer against KB-oral carvity cancer, NCI-H187 small cell lung cancer, and MCF7-Breast cancer. In addition, kaempferol (**7**) showed antioxidative activity (IC_{50} 9.67 ± 0.29 μM) that was three times stronger than that of ascorbic acid (IC_{50} 25.41 ± 0.92 μM). *trans*-Resveratrol (**14**) showed moderate antioxidative activity (IC_{50} 45.90 ± 0.22 μM), which was almost better than BHT (IC_{50} 46.56 ± 0.45 μM).

This information of phytochemistry and biological activities of isolated compounds are very important for development and apply into related fields, for example cosmetics, agricultures and pharmacy.

บทคัดย่อ

งานวิจัยนี้เป็นการศึกษาองค์ประกอบทางเคมีของดอก ใบ ราก ลำต้น และรากของชุมเห็ดเทศ (*Cassia alata* Linn.) โดยอาศัยเทคนิคทางโครมาโทกราฟีและการตกผลึก วิเคราะห์โครงสร้างของสารบริสุทธิ์ที่ได้ด้วยวิธีทางสเปกโทรสโกปี ได้แก่ UV IR และ NMR ส่วนสกัดหยาบและสารบริสุทธิ์ที่แยกได้นำมาศึกษาฤทธิ์ต้านแบคทีเรียแกรมบวก จำนวน 3 เชื้อ (*B. cereus S. aureus* และ MRSA SK1) และแบคทีเรียแกรมลบ จำนวน 3 เชื้อ (*E. coli P. aurenginosa* และ *S. typhimurium*) ด้วยวิธี broth microdilution ศึกษาฤทธิ์ต้านมะเร็งด้วยวิธี resazurin microplate assay และศึกษาฤทธิ์ต้านปฏิกิริยาออกซิเดชันด้วยวิธี DPPH assay

การศึกษาองค์ประกอบทางเคมีของส่วนสกัดหยาบของชุมเห็ดเทศ (*Cassia alata* Linn.) แยกสารได้ 23 สาร ได้แก่ hydroxyquinol (**1**) 2′,6′-dihydroxy-4′-methoxydihydrochalcone (**2**) stigmasterol (**3**) ziganein (**4**) aloe-emodin (**5**) emodin (**6**) kaempferol (**7**) diosmetin (**8**) physcion (**9**) *β*-sitosterol (**10**) lupeol (**11**) caffeic acid (**12**) apigenin (**13**) *trans*-resveratrol (**14**) ω-hydroxyemodin (**15**) orientalone (**16**) euxanthone (**17**) 3-geranyloxy-1,7-dihydroxyxanthone (**18**) *trans*-dihydrokaempferol (**19**) luteolin (**20**) lunatin (**21**) 7,4′-dihydroxy-5-methoxyflavone (**22**) และ hydroquinone (**23**) ในจำนวนสารที่แยกได้ทั้งหมดมีสารที่รายงานเป็นครั้งแรกของชุมเห็ดเทศ จำนวน 16 สาร ได้แก่ สาร **1 2 4 8 9 11-19 21** และ **22**

ผลการศึกษาฤทธิ์ต้านแบคทีเรียในเบื้องต้นของส่วนสกัดหยาบทั้งหมดพบว่าสามารถยับยั้งการเจริญของเชื้อแบคทีเรียแกรมบวกและแกรมลบได้ ซึ่งสอดคล้องกับฤทธิ์ต้านแบคทีเรียของสารบริสุทธิ์ **2** และ **6** สามารถยับยั้งการเจริญของเชื้อแบคทีเรีย *Bacillus cereus* สายพันธุ์ TISTR 687 และ methicillin resistant *Staphylococcus aureus* สายพันธุ์ SK1 ระดับดีมากด้วยค่า MICs เท่ากับ 8 และ 4 μg/mL ตามลำดับ ส่วนสกัดหยาบไดคลอโรมีเทนและอะซิโทนของลำต้นชุมเห็ดเทศไม่สามารถยับยั้งการเจริญของเซลล์มะเร็งช่องปาก KB-oral carvity เซลล์มะเร็งปอด NCI-H187 และเซลล์มะเร็งเต้านม MCF7 ได้ นอกจากนี้ยังพบว่า สาร **7** แสดงฤทธิ์ต้านปฏิกิริยาออกซิเดชัน (IC_{50} 9.67 ± 0.29 μM) ได้ดีกว่ากรดแอสคอบิค (IC_{50} 25.41 ± 0.92 μM) ถึง 3 เท่า และสาร **14** ยังแสดงฤทธิ์ต้านปฏิกิริยาออกซิเดชัน (IC_{50} 45.90 ± 0.22 μM) ได้ดีกว่า BHT (IC_{50} 46.56 ± 0.45 μM) อีกด้วย

ABSTRACT

This research involved the phytochemical investigation of flowers, leaves, roots, stems, and twigs of *Cassia alata* Linn. by chromatographic techniques and crystallization. All isolated pure compounds were characterization by UV, IR, and NMR spectroscopic methods. Antibacterial activity against three Gram-positive bacteria (*B. cereus*, *S. aureus*, MRSA SK1) and three Gram-negative bacteria (*E. coli*, *P. aurenginosa*, and *S. typhimurium*) were evaluated by broth microdilution method. Anticancer and antioxidation activities of the crude extracts and isolated pure compounds were evaluated using resazurin microplate assay and DPPH assay, respectively.

Phytochemical investigation of the extracts of *C. alata* Linn. yielded 23 compounds: hydroxyquinol (**1**), 2′,6′-dihydroxy-4′-methoxydihydrochalcone (**2**), stigmasterol (**3**), ziganein (**4**), aloe-emodin (**5**), emodin (**6**), kaempferol (**7**), diosmetin (**8**), physcion (**9**), *β*-sitosterol (**10**), lupeol (**11**), caffeic acid (**12**), apigenin (**13**), *trans*-resveratrol (**14**), ω-hydroxyemodin (**15**), orientalone (**16**), euxanthone (**17**), 3-geranyloxy-1,7-dihydroxyxanthone (**18**), *trans*-dihydrokaempferol (**19**), luteolin (**20**), lunatin (**21**), 7,4′-dihydroxy-5-methoxyflavone (**22**), and hydroquinone (**23**). Sixteen compounds of them (**1**, **2**, **4**, **8**, **9**, **11-19**, **21**, and **22**) were reported for the first time as metabolites of *C. alata*.

The antibacterial activity screening results, all crude extracts were able to inhibit the growth of gram positive and gram negative bacteria, according to the among the isolated compounds, compounds **2** and **6** exhibited a strong antibacterial activity against *Bacillus cereus* TISTR 687 and methicillin resistant *Staphylococcus aureus* -SK1 with the MICs values of 8 and 4 μg/mL, respectively. The dichloromethane and acetone extracts of *C. alata* stems showed inactive anticancer against KB-oral carvity cancer, NCI-H187 small cell lung cancer, and MCF7-breast cancer. Moreover, compounds 7 was found to exhibit antioxidative activity with IC_{50} value of 9.67 ± 0.29 μM that was three times stronger than that of ascorbic acid (IC_{50} 25.41 ± 0.92 μM). Compound **14** was also found to show more potent antioxidative activity (IC_{50} 45.90 ± 0.22 μM) than BHT (IC_{50} 46.56 ± 0.45 μM), respectively.

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ABBREVIATION AND SYMBOLS

Singlet	*s*
Doublet	*d*
Triplet	*t*
Quartet	*q*
Multiplet	*m*
Broadsinglet	*br s*
Doubletofdoublet	*dd*
Doubletoftriplet	*dt*
Kilogram	kg
Gram	g
Milligram	mg
Microgram	µg
Millimolar	mM
Micromolar	µM
Milliter	mL
Microliter	µL
Reciprocal Centimeter (wave number)	cm^{-1}
Hour	h
Minute	min
Percentage	%
Centimeter	cm
Millimeter	mm
Nanometer	nm
Melting Point	m.p.
Chemical Shift Relative to TMS	δ

ABBREVIATION AND SYMBOLS (continued)

Coupling Constant	*J*Molar
Extinction Coefficient	ε
Degree Celcius	°C
Megahertz	MHz
Part per Million	ppm
Concentration	c
Maximum Wavelength	λ_{max}
Infrared	IR
Ultraviolet-Visible	UV
Nuclear Magnetic Resonance	NMR
OneDimentional Nuclear Magnetic Resonance	1D NMR
Two Dimentional Nuclear Magnetic Resonance	2D NMR
Proton Nuclear Magnetic Resonance	^{1}H NMR
Carbon Nuclear Magnetic Resonance	^{13}C NMR
Correlated Spectroscopy	COSY
Distortionless Enhancement by Polarization Transfer	DEPT
Heteronuclear Multiple Quantum Correlation	HMQC
Heteronuclear Multiple Bond Correlation	HMBC
Column Chromatography	CC
Quick Column Chromatography	QCC
Preparative Thin-Layer Chromatography	PLC
Thin-Layer Chromatography	TLC
Tetramethylsilane	TMS
Deuterochloroform	$CDCl_3$
Deuteroacetone	Acetone-d_6
Deuterodimethylsulfoxide	DMSO-d_6

ABBREVIATION AND SYMBOLS (continued)

Tetrachloromethane	CCl_4
Dichloromethane	CH_2Cl_2
Chloroform	$CHCl_3$
Ethyl Acetate	EtOAc
Acetone	Me_2CO
Methanol	MeOH
Minimum Inhibition Concentrations	MICs
Mueller Hinton Agar	MHA
Mueller Hinton Broth	MHB
Normal Saline Solution	NSS
Colony Forming Unit	CFU
Revolutions per Minute	rpm
Dimethyl sulfoxide	DMSO
Bacillus cereus	B.C
Escherichia coli	E.C
Methicillin resistant *Staphylococcus aureus*	MRSA
Pseudomonas aurenginosa	Ps.A
Salmonellaetyphimurium	S.T
Staphylococcus aureus	S.A
Absorbance	Abs
% inhibition	% I
50% Inhibition Concentration	IC_{50}
ButylatedHydroxytoluene	BHT
1,1-Diphenyl-2-picrylhydrazyl	DPPH

CHAPTER 1
INTRODUCTION

1.1 Statement and Significance of the Problem

More than 36 million people died from non-communicable diseases in 2008, mainly cardiovascular diseases (48%), cancers (21%), chronic respiratory diseases (12%) and diabetes (3%) (World Health Organization, 2011). Plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contribution to human health and well being (Igbinosa, O. O., Igbinosa, O. E. & Aiyegoro, 2009). Developing countries still depend mainly on medicinal herbs due to their cheaper cost and their effectiveness in the treatment of various infectious diseases with lesser side effects and are widely accepted as sources of antioxidants substances (Joshi, Mishra, Khetwal & Bisht, 2012). With gradually increasing cases of human diseases all around microbes have also increased to a great extent. Although pharmacological industries have produced a number of new antibiotics in the last three decades, the resistance to these drugs by microorganisms has increased (Das & Choudhury, 2010). In recent times the critical area of primary health concern is the usual causative agents that are responsible for the incidence of new and re-emerging infectious diseases which pathogenic bacteria are frequently exposed to infection for example gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*, *Salmonella typhimurium*) (Joshi et al., 2012). The synthetic drug, antimicrobials of plant origin are not associated with many side effects and an enormous therapeutic potential to heal many infectious diseases (Iwu Duncan & Okunji, 1999). There is need to develop alternative microbial drugs which plants are also known to contain enumerable biological active compounds which posse's antibacterial properties (Anushia et al., 2009).

The diversities of plants in Thailand are found to possess several medicinal properties. Medicinal plants have been widely used in the treatment of illness and diseases for centuries. Pure compounds extracted from many plants and many parts of

the plants are explored and tested for biological activities. Plants of the family Leguminosae is the world's most important species because a large number of these families are used in Thai traditional medicine. They have been found to be a source of different secondary metabolites, flavonoids, anthraquinones, and xanthones with various biological activities. *Cassia alata* Linn. is one of a species in *Cassia* genus, family of Leguminosae. Therefore, *C. alata* was chosen for the phytochemical investigation as well as the evaluation of antibacterial, anticancer, and antioxidation activities of the crude extracts and the isolated compounds. The study of phytochemistry and biological activities are very important because the information from the study of bioactive compounds will be used for development and apply into related fields, for example cosmetics, agricultures and pharmacy.

1.2 Objectives

The objectives of this research involved the phytochemical investigation of *Cassia alata* Linn. and evaluation of antibacterial, anticancer and antioxidation activities of the crude extracts and isolated pure compounds.

1.3 Scope of Study

1.3.1 Extraction, isolation and purification of secondary metabolite from the flowers, leaves, roots, stems, and twigs of *Cassis alata* Linn. by chromatography and crystallization will be performed.

1.3.2 Characterization of all isolates by spectroscopic methods (UV, IR and NMR).

1.3.3 Evaluation of antibacterial activity against three Gram-positive bacteria (*B. cereus*, *S. aureus*, MRSA SK1) and three Gram-negative bacteria (*E. coli*, *P. aurenginosa*, and *S. typhimurium*) of crude extracts and pure compounds.

1.3.4 Evaluation of anticancer activity of crude extracts and pure compounds.

1.3.5 Evaluation of antioxidation activity of crude extracts and pure compounds using FIA method.

CHAPTER 2
LITERATURE REVIEWS

2.1 General Characteristics of *Cassia alata* Linn.

Cassia alata, belonging to Leguminosae family. It is a native of tropical America, now widespread over warm countries (Ross, 2003). In Thailand, it is commonly known as Chum-Hed-Thed (Gardner, Sidisunthorn & Anusarnsunthorn, 2000). Leaf is a simple pinnate, oblong, rounded at both ends, smooth and no glands. Flower is bright yellow, in upright spike-like cluster at the top of twigs. It is an individual flower, very short (2 to 4 mm) of stalks, ± 2 cm of petal and 2 stamens longer than others. Fruit is black pod of 10 to 20 x 1.5 to 2 cm size, flattened splitting with four wide ridges. Seed is tabular triangle of width 0.18-0.20 cm and length 0.40 - 0.42 cm. Stem is brown shrub stands 3-4 m tall (Gardner et al., 2000) as shown in figure 2.1.

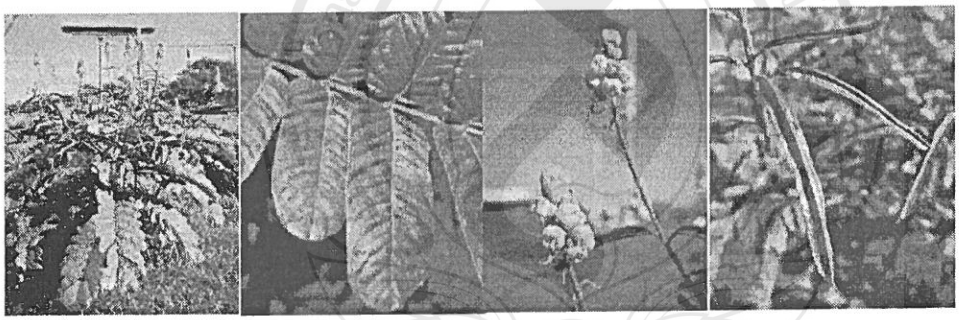


Figure 2-1 *Cassia alata* Linn.

2.2 Chemical Constituents Isolated from *Cassia alata* Linn.

According to Napralert database, Science direct and Chemical Abstracts, several types of compounds have been reported to be present in *Cassia* genus, such as coumarins, flavonoids, and steroids. Table 2-1 summarizes the chemical constituents which were reported from *C. alata* Linn.

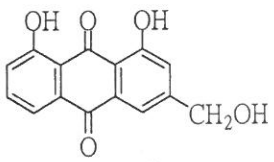
Table 2-1 Compounds Isolated from *Cassia alata* Linn.

Part	Compound (Structure)	Reference
Fruit	aloe emodin (1)	Rai, 1978
	emodin (2)	
	rhein (3)	
Leaf	aloe emodin (1)	Rao&Subhashini, 1986
	leucoanthocyanin (4)	Harrison &Garro, 1997
	aloe emodin-8-*O*-*β*-D-glucoside (5)	
	chrysophanic acid (6)	
	emodin (2)	
	astragalin (7)	Martin, Ohtani, Kasai &Yamasaki,1998
	kaempferol (8)	
	kaempferol-3-*O*-gentiobioside (9)	Yagi, El-Tigani& Adam, 1998
	rhein (3)	
Root	alquinone (10)	Yadav&Kalidhar, 1994
Seed	chrysoeriol-7-*O*-(2"-*O*-*β*-D-manno-pyranosyl)-*β*-D-allopyranoside (11)	Gupta & Singh, 1991
	glycerol (12)	
	rhamnetin-3-*O*-(2"-*O*-*β*-D-manno-pyranosyl)-*β*-D-allopyranoside (13)	
	erythritol (14)	Singh, 1998
Seed oil	campesterol (15)	Miralles&Gaydou, 1986
	22-dihydrospinasterol (16)	
	28-isoavenasterol (17)	
	linoleic acid (18)	
	oleic acid (19)	
	palmitic acid (20)	
	β-sitosterol (21)	
	stigmasterol (22)	

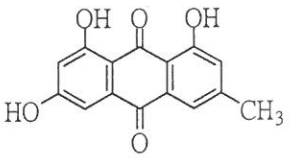
Table 2-1 (continued)

Part	Compound (Structure, Type)	Reference
Stem	dalbergin (23)	Hemlata&Kalidhar, 1993
	daucosterol (24)	
	emodin (2)	
	luteolin (25)	
	santal (26)	
	β-sitosterol (21)	
	alarone (27)	Hemlata&Kalidhar, 1994
	alatonal (28)	
	1,5-dihydroxy-2-methylanthraquinone (29)	
	2,6-dimethoxybenzoquinone (30)	Rai& Prasad, 1994
	5-hydroxy-2-methylanthraquinone (31)	

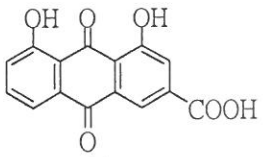
Structure of Compounds Isolated from *Cassia alata* Linn.



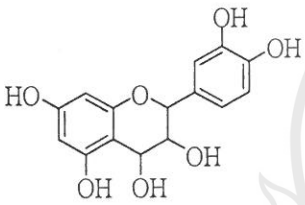
1 : aloe emodin



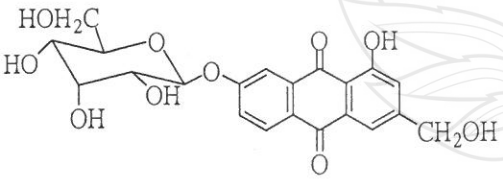
2 : emodin



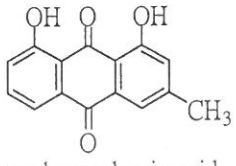
3 : rhien



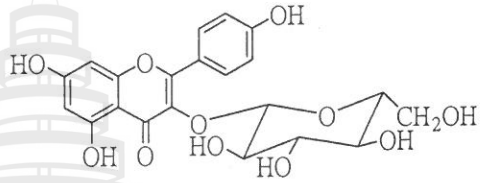
4 : leucoanthocyanin



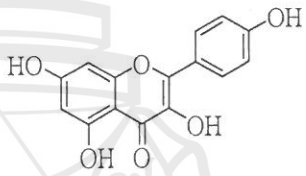
5 : aloe emodin-8-*o*-β-D-glucoside



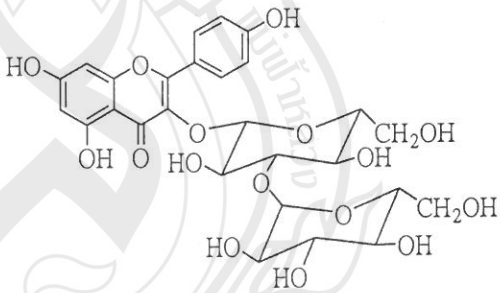
6 : chrysophanic acid



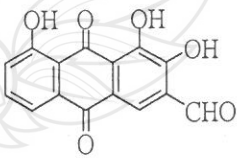
7 : astragalin



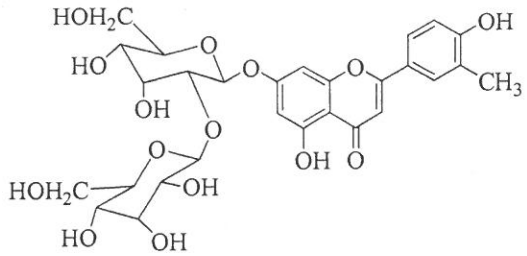
8 : kaempferol



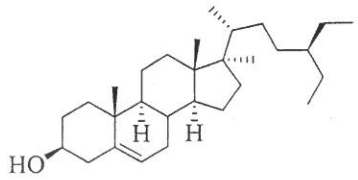
9 : kaempferol-3-*o*-gentiobioside



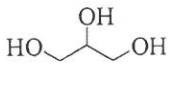
10 : alquinone



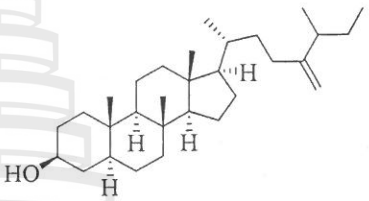
11: chrysoeriol-7-*O*-(2"-*O*-*β*-D-manno-pyranosyl)-*β*-D-allopyranoside



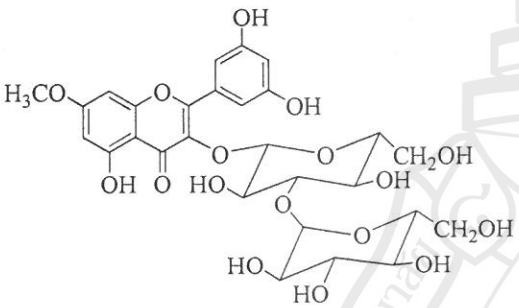
16: 22-dihydrospinasterol



12: glycerol

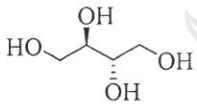


17: 28-isoavenasterol



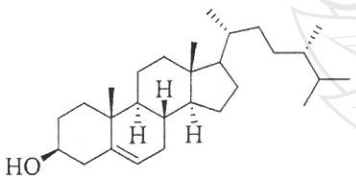
13: rhamnetin-3-*O*-(2"-*O*-*β*-D-manno-pyranosyl)-*β*-D-allopyranoside

18: linoleic acid



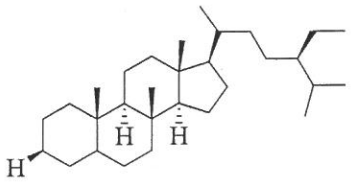
14: erythritol

19: oleic acid

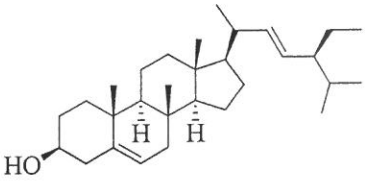


15: campesterol

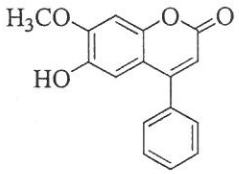
20: palmitic acid



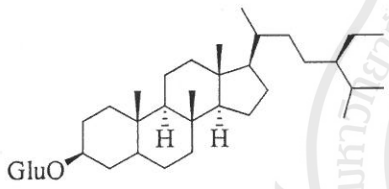
21:β-sitosterol



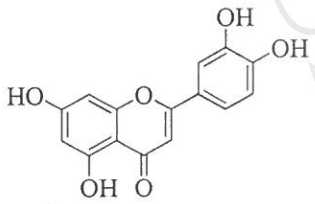
22: stigmasterol



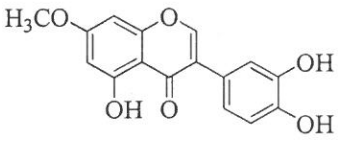
23: dalbergin



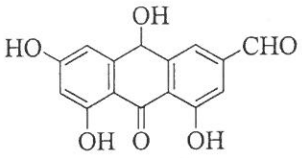
24: daucosterol



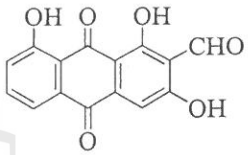
25: luteolin



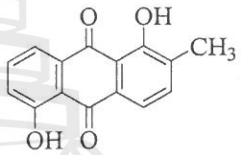
26: santal



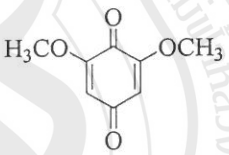
27: alarone



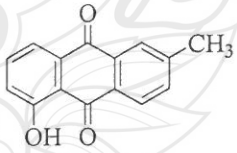
28: alatonal



29: 1,5-dihydroxy-2-methylanthraquinone



30: 2,6-dimethoxybenzoquinone



31: 5-hydroxy-2-methylanthraquinone

2.3 Biological Activities of *Cassia alata* Linn.

Cassia genus, belonging to Leguminosae family. The plant in this genus generally produce a variety of secondary metabolites including anthraquinones, alkaloids, flavonoids, pyrrolizine and pyrrolizidine alkaloids, triterpenes, steroids and tannins (Hemlata and Kalidhar, 1993; Miralles and Gaydou, 1986; Idu *et al.*, 2007) some of which possess interesting biological and pharmacological activities, such as antimicrobial, antioxidative, anti-inflammatory, antitumor as well as cytotoxic activities (Ibrahim and Osman, 1995; Panichayupakaranant and Kaewsuwan, 2004; Fernand *et al.*, 2008). Due to these properties, *Cassia* species has attracted attention as important sources for medicinal treatment. It has been used to treat eczema, itching and skin infection in human (Palanichamy and Nagarajan, 1990). *Cassia alata* Linn., locally known in Thai as Chum-Hed-Thed. It was observed that methanol extracts of leaves, flowers, stem and root barks of *C. alata* shown to have a broad spectrum of antibacterial activity (Khan *et al.*, 2001). On the basis of DPPH radical scavenging assay-guided isolation, kaempferol from *C. alata* leaves exhibited antioxidant activity with ED_{50} 9.99 >M that was six times stronger than that of BHT with ED_{50} 57.41 >M and fifty eight times stronger than that of emodin with ED_{50} 578.87 >M (Panichayupakaranant and Kaewsuwan, 2004). Previous research studies have led to the isolation of a number of constituents with many biological activities, making these potential agents for the treatment of diseases (Gurib-Fakim, 2006). In a continuing search for bioactive metabolites from *C. alata*, we now report the result of a phytochemical investigation of *C. alata* (leaves, roots and twigs) yielding thirteen compounds. Moreover, this work demonstrates that *C. alata* is among the potential sources of antibacterial and antioxidative compounds.

2.3.1 Antimicrobial Activities of *Cassia alata* Linn.

The ethanolic extract of *C. alata* have been reported to show high activity against against *Trichophyton mentagorphytes* var. *interdigitale*, *Trichophyton mentagrophytes* var. *mentagorophytes*, *Trichophyton rubrum* and *Microsporum gypseum* with MIC value of 125 mg/mL, whereas *Microsporum canis* was 62.5 mg/mL (Ibrahim & Osman, 1995). The methanolic extracts of *C. alata* leaves,

flowers, barks and roots at the concentration of 4 mg/mL inhibited many types of bacteria including *Escherichia coli* and *Staphylococcus aureus* (Khan et al., 2001). The ethanolic and water extracts of leaves and barks inhibited the growth of *E.coli* (Somchit, Reezal, Nur & Mutalib, 2003). The leaves extract of *C. alata* exhibited higher activity gainst *T. rubrum* and *M. gypseum*than than the leaves extract of *Cassia fistula* and *Cassia tora* with the IC_{50} of hyphal growth at 0.5 and 0.8 mg/mL, respectively (Phongpaichit, Pujenjob, Rukachaisirikul & Ongsakul, 2004). Kaempferol and aloe-emodin were showed the most active compounds against MRSA with MICs value of 13.0±1.5 and 12.0±1.5 μg/mL, respectively (Hazni, Ahmad, Hitotsuyanagi, Takeya & Chee-Yan, 2008). Aloe-emodin was already known to be the most active anthraquinone derivative from *C. alata* against some dermatophytic fungi (Fuzellier, Mortier & Lectard, 1982). The ethanolic extract of leaves exhibited the inhibition zone against *Trichophyton verrucosum* and *Epidermophyton floccosum* of 20.50 and 20.00 mm, respectively (Sule et al., 2010).

2.3.2 Anticancer Activity of *Cassia alata* Linn.

The aqueous extracts of leaves of *C. alata* were used to treat eczema, itching and skin infections in humans (Palanichamy & Nagarajan, 1990; Morah & Otumu, 1991). A variety of biological activities including anticancer activity, rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid), the primary anthraquinone in the roots of *C .alata* is one of the major bioactive compounds (Fernand et al., 2008). Rhein has been investigated as a potential inhibitor of cancer cell viability and the mechanisms by which rhein inhibits cancer cell viability have been reported to include induction of apoptosis (Lin, Chen, Huang & Wang, 2007), against angiogenesis and breast cancer cell viability (Fernand et al., 2008).

2.3.3 Antioxidation Activity of *Cassia alata* Linn.

Panichayupakaranant & Kaewsuwan, 2004 reported that the methanolic extract exhibited antioxidant activity (ED_{50} 9.99 μM) that was six times stronger than that of BHT (ED_{50} 57.41 μM) and fifty eight times stronger than that of emodin (ED_{50}578.87 μM). Olarte, Herrera, Villasenñor & Jacinto, 2010 reported that a new indole alkaloid, 1-(4′-hydroxyphenyl)-2,4,6-trihydroxy-indole-3-carboxylic acid,

which contain in the EtOAc fraction of the leaf extract from *C. alata* demonstrated a dose-dependent scavenging activity against DPPH with an IC_{50} of 0.0311 μM ± 0.002, indicating strong antioxidant potential.

Previous research studies have shown that the *C. alata* is a tree of interest, since various plant extracts displayed many biological activities and have been used for treatment diseases. Therefore, we are interested in phytochemical investigation of this plants and evaluation of antibacterial, anticancer and antioxidation activities of the isolated compounds.

CHAPTER 3
METHODOLOGY

3.1 General

Melting points were measured on a BÜCHI B-540 melting point apparatus. It was recorded in °C. Ultraviolet spectra (UV) were recorded using UV-Vis spectrometer (PerkinElmer Lambda, USA). Principle bands (λ_{max}) were recorded as wavelengths (nm) and log ε in methanol solution. Infrared spectra (IR) were recorded on Perkin-Elmer FTSFT IR/Spectum spectrometer at United States of America. Major bands (ν_{max}) were recorded in wavenumber (cm^{-1}). 1D and 2D NMR spectra were performed on a Bruker AVANCE 300 MHz at Germany (Silpakorn University, Nakhon Pathom) , Brüker FTNMR Ultra Shield 300 MHz at Germany (Prince of Songkla University, Songkhla), Brüker FTNMR Ultra Shield 400 MHz at Germany (Naresuan University, Phitsanulok), and Varian INOVA 500 MHz at Germany (Chulalongkorn University, Bangkok). Spectra were recorded in $CDCl_3$ or acetone-d_6 solution and recorded as chemical shift (δ) value in ppm down field from TMS (internal standard δ 0.00). Pre-coated TLC aluminum sheets of silica gel 60 F_{254} (20x20 cm, layer thickness 0.2 mm, Merck, Germany) were used for analytical purposes and the compounds were visualized under ultraviolet light or anisaldehyde-sulfuric acid and vanillic acid reagents. Preparative thin-layer chromatography (PLC) was carried out on glass plates coated with silica gel 60 F_{254} (20x20 cm, layer thickness 1.0 mm, Merck, Germany). Bands were detected by exposure to short wavelength UV light. Column chromatography (CC) and quick column chromatography (QCC) were performed on silica gel 100 (0.063-0.200 mm, Merck, Germany) and silica gel 60 (0.063-0.230 mm, Merck, Germany), respectively. Organic solvents for extraction and chromatography (hexanes, dichloromethane, chloroform, Ethyl acetate, acetone, and methanol, commercial grade) were distilled at their boiling point ranges prior to use. Solvents for UV and IR were analytical grade reagent (Merck, Germany). The analytical grade of absoluteethanol, 1,1-diphenyl-2-picrylhydrazyl free radical ($DPPH^{\bullet}$, Fluka, USA), ascorbic acid (Fluka, USA) and butylated hydroxytoluene (BHT, Fluka, USA) were used for antioxidative activity

testing and the absorption of the test solution were measured with spectrophotometer (Thermo/Genesys 20). The dimethyl sulfoxide (DMSO) and nutrient broth were used for antibacterial activity testing against 6 strains of microorganism; *Bacillus cereus* TISTR 687, *Escherichia coli* TISTR 780, *Pseudomonas aeruginosa* TISTR 781, *Salmonella typhimurium* TISTR 292, *Staphylococcus aureus* TISTR 1466, and methicillin resistant *Staphylococcus aureus* SK1. Vancomycin and gentamicin were used as standard makers of antibacterial activity.

3.2 Plant and Microorganism Culture Materials

Flowers, leaves, roots, stems and twigs of *C. alata* Linn. were collected from Nong Khai Province, North eastern of Thailand, in December, 2009. The plant was identified by Mr. James Maxwell, Chiang Mai University Herbarium and the specimen (957) was deposited at Chiang Mai University herbarium, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand.

Five microorganism cultures (*B. cereus* TISTR 687, *E. coli* TISTR 780, *Ps. aurenginosa* TISTR 781, *S. typhimurium* TISTR 292 and *S. aureus* TISTR 1466) were purchased from the Microbiological Resources Centre of the Thailand Institute of Scientific and Technological Research and kept as stock cultures at the Microbiology Laboratory at Mae Fah Luang University. Methicillin resistant *S. aureus* (MRSA)-SK1 was supported by Department of Microbiology, Faculty of Science, Prince of Songkla University.

KB-oral carvity cancer, NCI-H187 small cell lung cancer, and MCF7-Breast cancer were tested for anticancer activity evaluation by National Center for Genetic Engineering and Biotechnology (BIOTEC).

3.3 Preparation of *Cassia alata* Extracts

The dried flowers, leaves, roots, stems, and twigs of *C. alata* Linn. were chopped into a small pieces and extracted with organic solvents at room temperature as follow:

Flowers (361.340 g) were extracted with CH_2Cl_2 (8 L, 5 days), acetone (8 L, 5 days) and methanol (8 L, 5 days), respectively, to give, after evaporation, the CH_2Cl_2

extract (crude FD, 2.250 g), the acetone extract (crude FA, 10.330 g) and the methanolic extract (crude FM, 1.720 g). Leaves (267.330 g) were extracted with CH_2Cl_2 (7 L, 7 days) and acetone (6 L, 7 days), respectively. Removal of the solvents from each extract under reduced pressure gave the CH_2Cl_2 (crude LD, 23.000 g) and acetone extracts (crude LA, 20.320 g). Roots (6.735 kg) were extracted with Me_2CO (31 L, 7 days) to give, after evaporation, the acetone extract (crude RA, 42.920 g). Stems (4.620 kg) were extracted with CH_2Cl_2 (28 L, 7 days) and Me_2CO (22 L, 10 days), respectively, to give, after evaporation, the CH_2Cl_2 (crude SD, 20.360 g) and acetone (crude SA, 40.460 g) extracts. Twigs (2.030 kg) were extracted with CH_2Cl_2 (12 L, 7 days) and Me_2CO (11 L, 7 days), respectively, to give, after evaporation, the CH_2Cl_2 (crude TD, 13.800 g) and acetone (crude TA, 15.850 g) extracts.

3.4 Isolation and Purification of *Cassia alata* Extracts

Crude FD (2.250 g) was subjected to CC (1.770 g) over silica gel eluted with hexanes-CH_2Cl_2, CH_2Cl_2 and CH_2Cl_2-Me_2CO. The collected fractions were combined according to the characteristic on TLC and evaporated to give fractions FD1-FD8. The selected fractions were further purified to give compounds **1-5**, as shown in figure 3-1. Fraction FD1 (yellow solid, 0.115 g) was purified by CC and eluted with 70% CH_2Cl_2-hexanes to give subfractions FD1.1-FD1.3. Subfraction FD1.1 was recrystallized from 80% CH_2Cl_2-hexanes to give **3** (25.5 mg) as a white solid. Fraction FD3 (yellow viscous liquid, 0.011g) was separated by CC using 10% EtOAc-hexanes to give subfractions FD3.1-FD3.3. Subfraction FD3.1 was further purified by CC and eluted with 2% EtOAc-hexanes to yield **4** (5.0 mg) as a yellow solid. Fractions FD5 (yellow solid, 0.008 g) and FD6 (yellow solid, 0.111 g) were purified by CC and eluted with 5% Me_2CO-CH_2Cl_2 to give an orange solid **5** (5.0 and 31.5 mg). Fractions FD7 (brown viscous liquid, 0.230 g) and FD8 (brown viscous liquid, 0.050 g) were purified by CC and eluted with 15%-20% Me_2CO-CH_2Cl_2 to yield **1** (84.2 mg) and **2** (15.3 mg) as a brown solid, respectively.

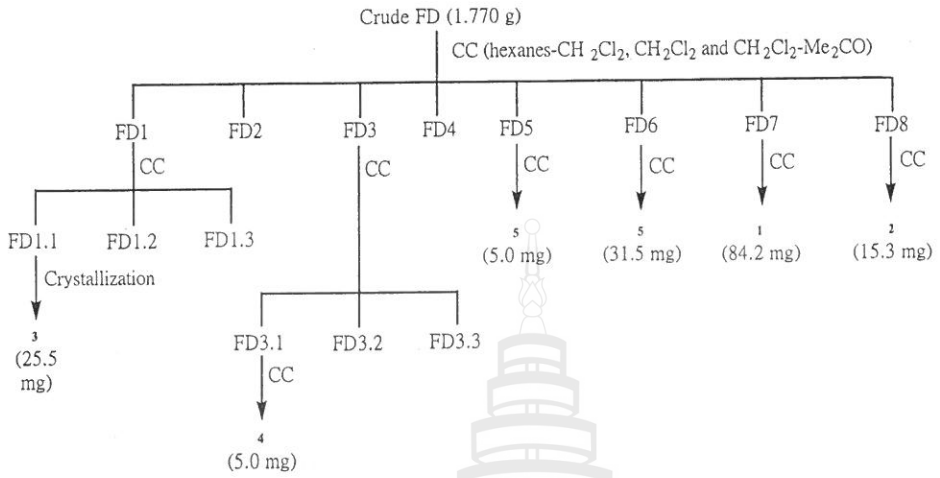


Figure 3-1 Isolation of Pure Compounds from Crude FD

Crude FA (10.330 g) was subjected to QCC (7.790 g) over silica gel eluted with hexanes, hexanes-EtOAc, and EtOAc to give fractions FA1-FA15. Fractions FA7 and FA8 were combined (greenish yellow viscous liquid, 0.096 g) and purified by CC (10%-15% EtOAc-hexanes) to give **5** as an orange solid (11.5 mg). Fraction FA10 (dark green solid, 0.068 g) was purified by CC (10%-20% EtOAc-hexanes) to give a yellow solid **5** (15.7 mg) and **6** (1.5 mg). Fraction FA11 (brown viscous liquid, 0.160 g) was purified by CC (15%-20% EtOAc-hexanes) to give an orange of **5** (12.6 mg). Fractions FA13 and FA14 (brown viscous liquid, 0.1434 g) were combined and purified by CC (20%-45%EtOAc-hexanes and 30% EtOAc-hexanes) to yield an orange solid **5** (1.9 mg), as shown in figure 3-2.

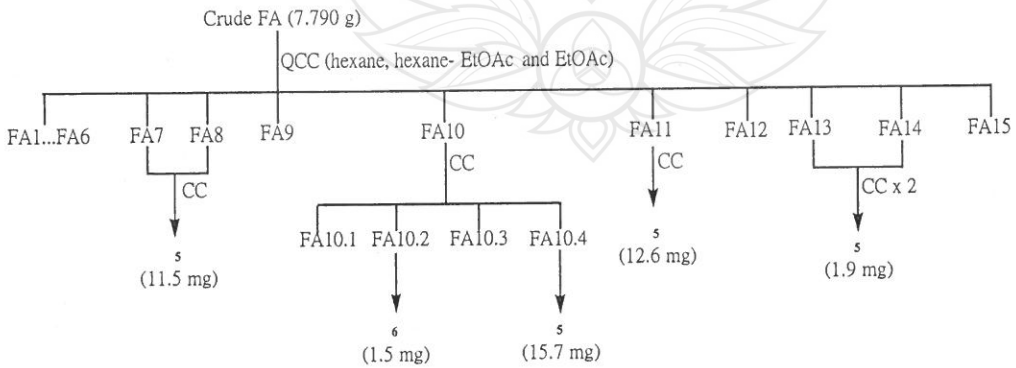


Figure 3-2 Isolation of Pure Compounds from Crude FA

Crude LD (23.000 g) was subjected to QCC over silica gel and gradiently eluted with hexanes, hexanes-CH_2Cl_2 and CH_2Cl_2. The collected fractions were combined according to the characteristic on TLC and evaporated to give fractions LD1-LD7. Fraction LD7 (dark brown viscous liquid, 4.206 g) was subjected to CC using 10%-25% EtOAc-hexanes to give fractions LD7.1-LD7.14. Subfraction LD7.10 (0.312 g) was purified by CC using CH_2Cl_2 then recrystallized (100% CH_2Cl_2) to yield **5** (3.2 mg) as a yellow solid.

Crude LA (20.320 g) was separated by QCC (15.880 g) using hexanes-CH_2Cl_2, CH_2Cl_2 and CH_2Cl_2-Me_2CO in a polarity gradient manner. The collected fractions were combined according to the characteristic on TLC to give fractions LA1-LA9. Fraction LA1 (brown viscous liquid, 0.055 g) was purified by CC eluted with 30%-60% EtOAc-hexanes to give **5** (1.4 mg) as a yellow solid. Fractions LA3 and LA4 were combined (brown solid, 0.097 g), purified by CC (20% Me_2CO-hexanes) to give an orange solid **5** (17.8 mg) and **6** (20.7mg). Fraction LA5 (orange solid, 0.138 g) was purified by CC (30%-40% EtOAc-hexanes) to give total an orange solid **5** (63.3 mg). Fraction LA8 (brown solid, 3.860 g) was purified by CC (50%-90% EtOAc-hexanes) then recrystallized in 65% EtOAc-hexanes to give a yellow solid **7** (150.1 mg). The filtrate mother liquid (0.610 g) and fraction LA8.3 were combined (fLA8.3, 1.267 g) and further purified by CC (eluted with 50% EtOAc-hexanes) to give subfractions fLA8.3.1 and fLA8.3.2. The second subfraction (0.666 g) was separated by CC (50% EtOAc-hexanes) then CC (30%-40% EtOAc-hexanes) to give **5** (1.4 mg) as an orange solid. Subfraction fLA8.3.2.3 (25.9 mg) was purified by CC (100% CH_2Cl_2) to give **8** (1.2 mg). Subfraction fLA8.3.2.5 was purified by CC (10%-15% Me_2CO-CH_2Cl_2) to give **7** (274.8 mg) as a yellow solid. Fraction fLA8.3.2.6 was purified by CC (10%-60% EtOAc-hexanes) to give **7** (54.1 mg) as a yellow solid as shown in figure 3-3.

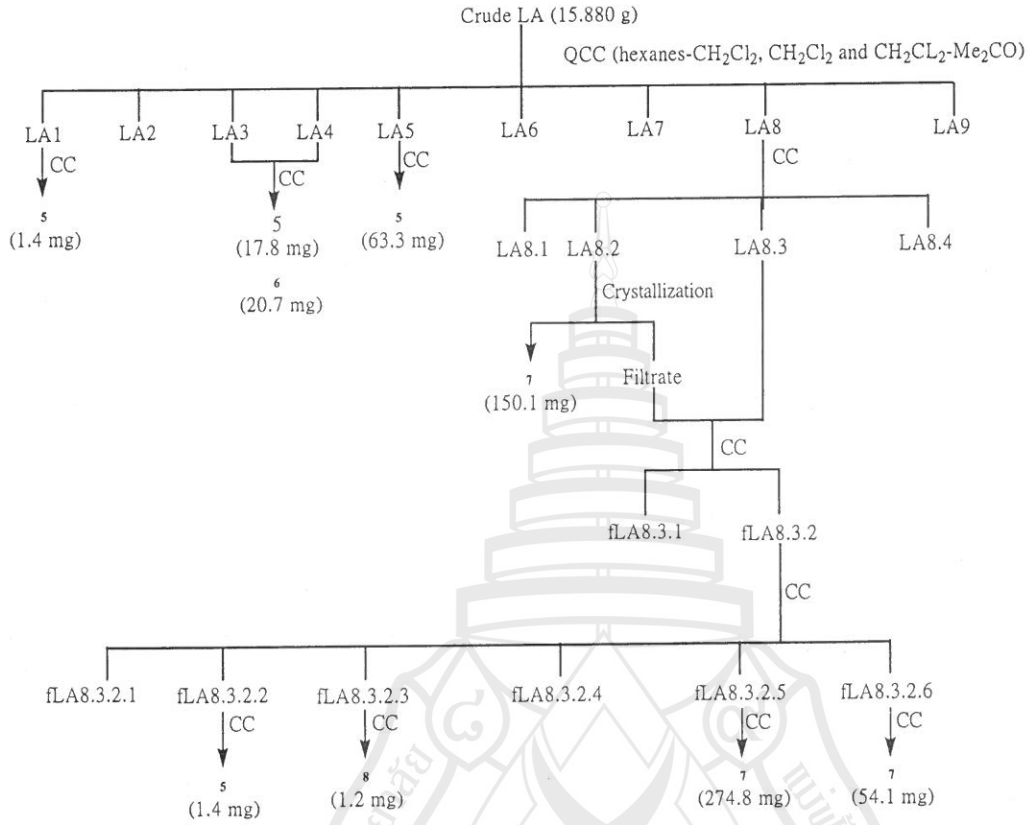


Figure 3-3 Isolation of Pure Compounds from Crude LA

A portion of crude RA (40.420 g) was subjected to QCC over silica gel eluted with hexanes, hexanes-CH_2Cl_2, CH_2Cl_2, CH_2Cl_2-Me_2CO, Me_2CO, and Me_2CO-MeOH. The collected fractions were combined according to the characteristic on TLC and evaporated to give fractions RA1-RA20. The selected fractions were further purified (figure 3-4) to give 12 compounds (**3**, **4**, **6**, and **8-16**).

Fraction RA2 (orange solid, 26.4 mg) was purified by CC eluted with hexanes and 5% CH_2Cl_2-hexanes to give an orange solid **4** (10.5 mg). Fraction RA3 (orange soild, 1.107 g) was purified by CC (hexanes) to give RA3.1-RA3.5. Fraction RA3.3 (191.0 mg) was rechromatographed on CC (hexanes-CH_2Cl_2) to give subfractions RA3.3.1-RA3.3.4. The third subfraction was purified by CC (100% hexanes and 10%-20% CH_2Cl_2-hexanes) to give an orange solid **4** (8.5 mg) and **9** (5.6 mg). Crystallization (1% Me_2CO-CH_2Cl_2) of the last subfraction to give **9** as an orange

soild, 5.3 mg. Subfraction RA3.4 (83.2 mg) was rechromatographed on CC (100% hexanes to10% CH_2Cl_2-hexanes) to yield **4** (2.5 mg) and **9** (12.4 mg) as an orange solid. Fraction RA4 (orange solid, 0.170 g) was separated on CC (hexanes and hexanes-CH_2Cl_2) to yield an orange solid **4** (2.5 mg) and **9** (35.0 mg). Fraction RA5 (orange solid, 0.204 g) was separated by CC (hexanes-CH_2Cl_2) to give an orange solid **4** (3.5 mg) and a yellow solid **9** (39.4 mg). Fraction RA7 (brown solid, 1.529 g) was purified on CC (hexanes-CH_2Cl_2, CH_2Cl_2-Me_2CO in a gradient manner) to give fractions RA7.1-RA7.4. Fraction RA7.2 (950.2 mg) was purified on CC (20%-40% Me_2CO-hexanes) to give **3** (20.2 mg) as a yellow viscous liquid and **16** (2.4 mg) as a yellow solid. Fraction RA8 (brown solid, 2.452 g) was separated by CC (hexanes-CH_2Cl_2, CH_2Cl_2-Me_2CO) to yield subfractions RA8.1-RA8.6. Subfraction RA8.2 (73.0 mg) was applied on PLC (20% Me_2CO-hexanes) to give **3** as a yellow viscous liquid (23.8 mg). Subfraction RA8.3 (651.3 mg) was crystallized from hexanes to obtain a white solid **10** (61.8 mg). The filtrate (467.1 mg) was purified by CC (5%-20% EtOAc-hexanes) then PLC (80% CH_2Cl_2-hexanes and 100% CH_2Cl_2) to give **16** (1.8 mg) as a yellow solid. Fraction RA9 (brown solid, 1.029 g) was separated by CC (10%-40% Me_2CO-hexanes) to give a white solid (**11**, 11.1 mg) and an orange solid (**6**, 93.3 mg and **14**, 14.3 mg) and **12** as a brown solid (3.3 mg). Fraction RA13 (brown solid, 2.370 g) was separated by CC (15%-50% Me_2CO-hexanes) to give **8** as a yellow solid (1.5 mg). Fraction RA14 (brown solid, 3.920 g) was subjected to QCC eluted with CH_2Cl_2 and CH_2Cl_2-Me_2CO to give fractions RA14.1-RA14.4. Fraction RA14.3 (3.336 g) was purified by CC (hexanes and Me_2CO) to yield total **15** (a yellow solid, 27.4 mg), **13** (a pale yellow solid, 3.0 mg) and **14** (a pale yellow solid, 589.6 mg) as shown in figure 3-4.

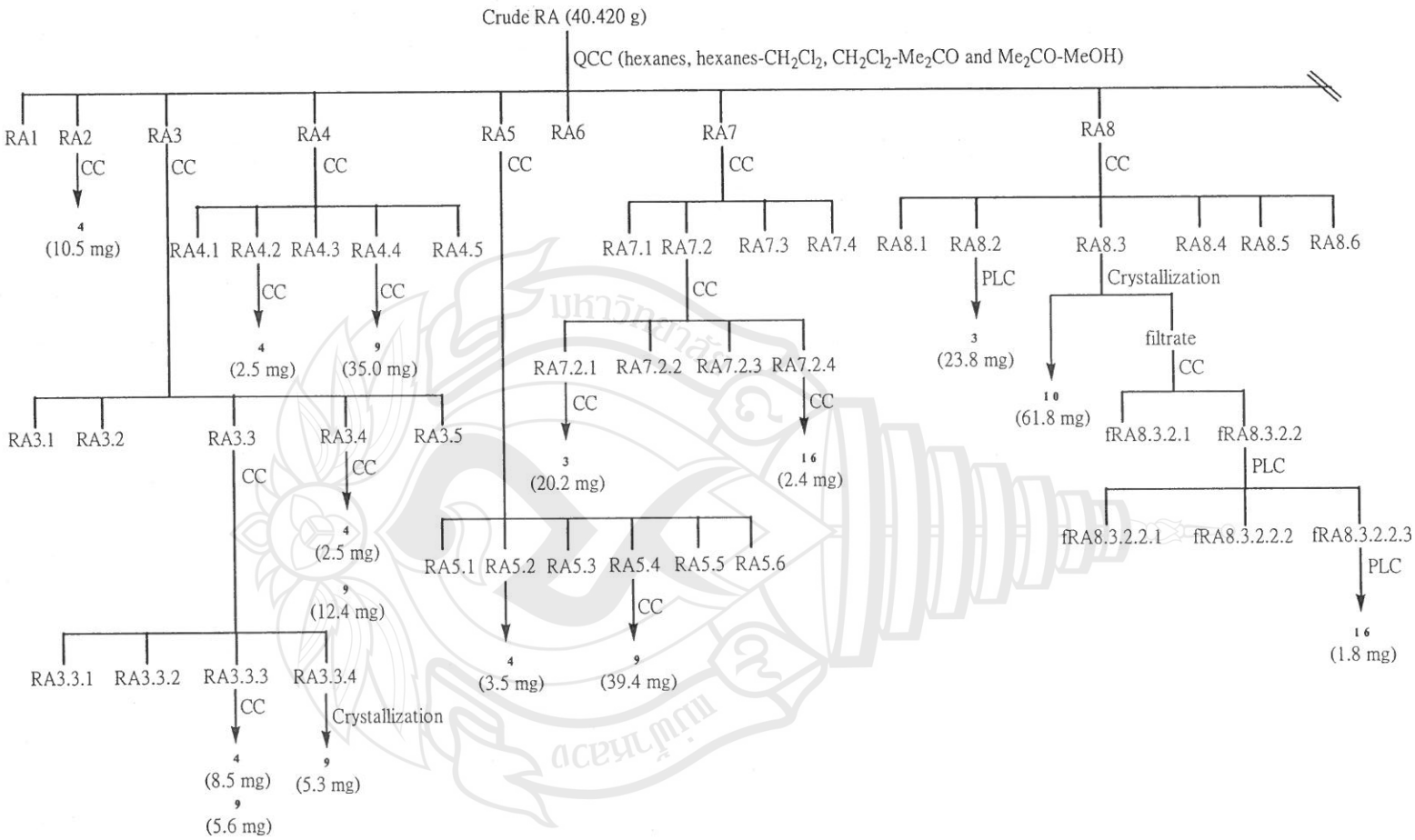


Figure 3-4 Isolation of Pure Compounds from Crude RA

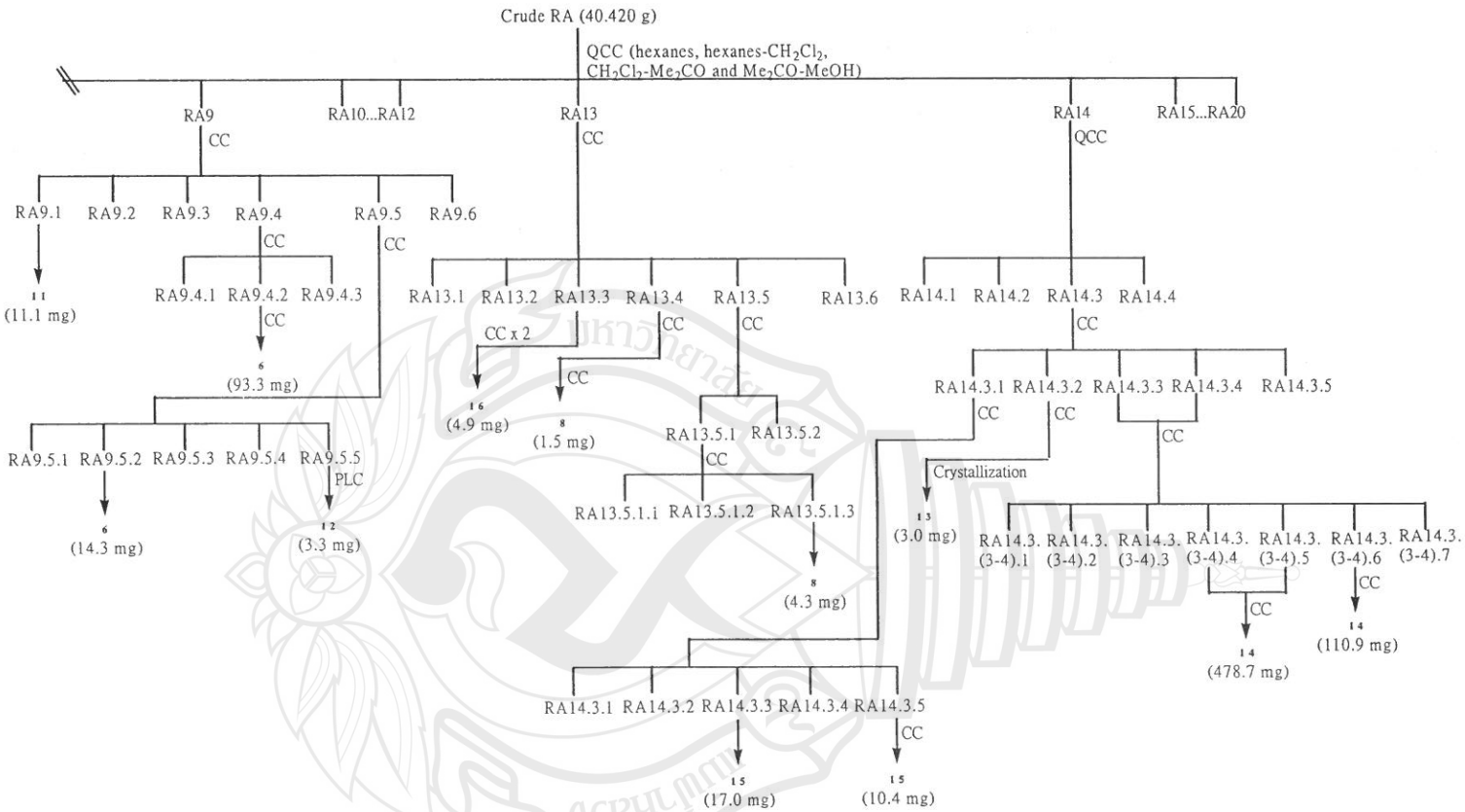


Figure 3-4 (continued)

Crude SD (20.360 g) was subjected to CC (16.880 g) over silica gel eluted with hexanes-CH_2Cl_2. The collected fractions were combined according to the characteristic on TLC and evaporated to give fractions SD1-SD27. Fraction SD4 (yellow viscous liquid, 0.087 g) was purified by CC (hexanes-CH_2Cl_2) to give a yellow solid **17** (3.5 mg). Fraction SD12 (dark orange solid, 0.054 g) was purified by CC (hexanes and hexanes-CH_2Cl_2) to give subfractions SD12.1-SD12.13. Subfraction SD12.4 (30.2 mg) was further purified by PLC (100% CH_2Cl_2) to give an orange solid (**4**, 25.6 mg). An orange solid of subfraction SD12.7 was **4** (2.5 mg) whereas a yellow solid of subfraction SD12.11 was **9** (3.5 mg) as shown in figure 3-5.

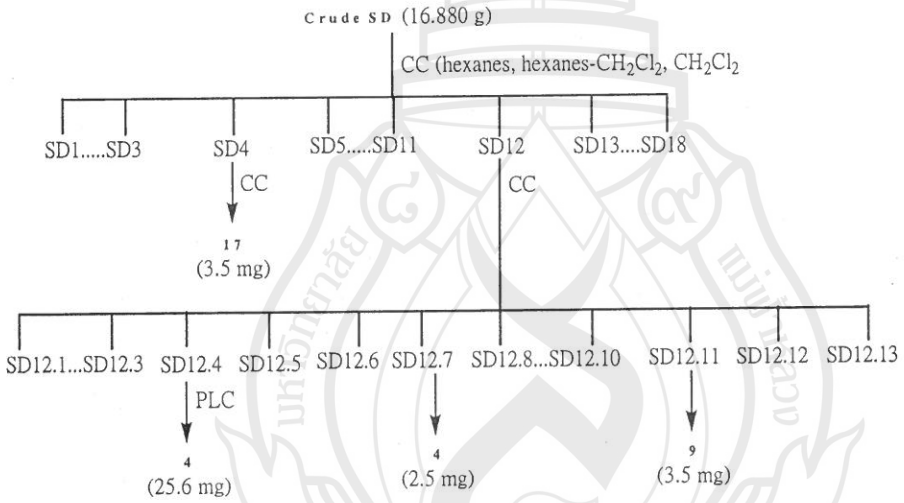


Figure 3-5 Isolation of Pure Compounds from Crude SD

Crude SA (40.460 g) was subjected to QCC (24.980 g) over silica gel and gradiently eluted with hexanes, hexanes-CH_2Cl_2, CH_2Cl_2, CH_2Cl_2-Me_2CO and Me_2CO. The collected fractions were combined to give fractions SA1-SA11. Fraction SA3 (orange viscous liquid, 0.096 g) was separated by QCC (100% hexanes up to 50% CH_2Cl_2-hexanes) to give an orange solid (**6**, 9.3 mg) and a pale yellow solid (**18**,10.0 mg). Fraction SA4 (orange viscous liquid, 0.018 g) was purified by CC (5-10% CH_2Cl_2-hexanes) to give an orange solid of **6** (2.6 mg) and **9** (3.6 mg). Fraction SA5 (orange viscous liquid, 0.042 g) was purified by CC (10%-60% CH_2Cl_2-hexanes) to yield an orange solid **4** (5.6 mg). Fraction SA6 (orange viscous liquid, 0.059 g) was

separated by CC (20%-40% CH_2Cl_2-hexanes) to give **9** (2.5 mg) as a colorless viscous liquid. Fraction SA8 (orange viscous liquid, 0.092 g) was purified by CC (30%-60% CH_2Cl_2-hexanes) to give **4** (yellow solid, 4.2 mg), **6** (orange solid, 8.8 mg) and **9** (orange solid, 6.0 mg). Fraction SA10 (orange viscous liquid, 0.027 g) was isolated by CC (30% CH_2Cl_2-hexanes) to yield an orange solid **14** (5.1 mg) as shown in figure 3-6.

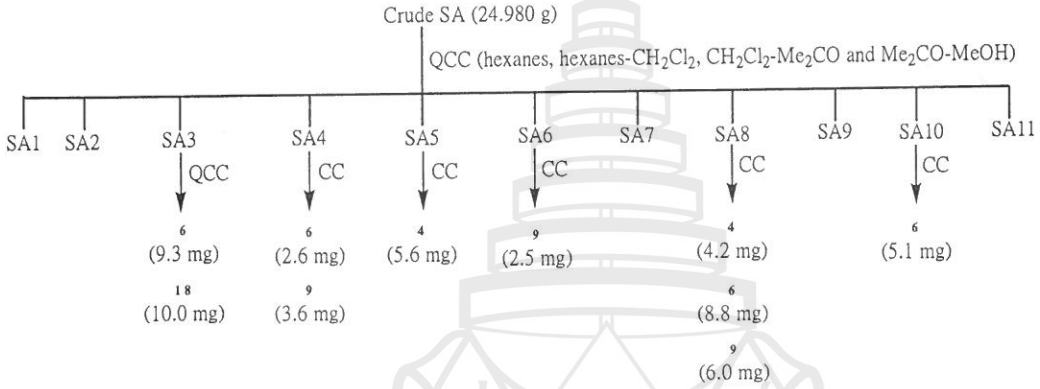


Figure 3-6 Isolation of Pure Compounds from Crude SA

Crude TD (13.800 g) was subjected to CC (8.057 g) over silica gel eluted with hexanes-Me_2CO. The collected fractions were combined to give fractions TD1-TD10. Fraction TD6 (orange viscous liquid, 0.297 g) was purified by CC (2% EtOAc-hexanes) to give an orange solid of **4** (43.7 mg). Fraction TD7 (dark orange viscous liquid, 0.297 g) was purified by CC (20%-25% CH_2Cl_2-hexanes) to give **4** (17.8.0 mg) as an orange solid. Fraction TD9 (brown viscous liquid, 0.257 g) was purified by crystallization (100% hexanes) to give **6** (4.0 mg) as an orange solid.

Crude TA (15.850 g) was subjected to QCC (10.300 g) over silica gel and gradiently eluted with hexanes-CH_2Cl_2, CH_2Cl_2, CH_2Cl_2-Me_2CO and Me_2CO. The collected fractions were combined according to the chromatogram on TLC to give fractions TA1-TA10. Fraction TA4 (brown solid, 0.443 g) was crystallization in CH_2Cl_2 yielded an orange solid **6** (72.0 mg). Fraction TA5 (brown solid, 0.222 g) was purified by CC (CH_2Cl_2) to give **6** (3.0 mg). Fraction TA7 (brown solid, 1.312 g) was separated by CC (5%-40% Me_2CO-CH_2Cl_2) to give subfractions TA7.1-TA7.11. Subfractions TA7.3 and TA7.4 were combined (0.142 g) and purified by CC (CH_2Cl_2)

to give a yellow solid of **13** (11.3 mg). Subfraction TA7.5 (25.5 mg) was separated by CC (CH_2Cl_2) and CC (30%-40% Me_2CO-hexanes) to give **13** (2.8 mg) as a yellow solid. Subfraction TA7.7 (0.263 g) was purified by CC and eluted with a step gradient of CH_2Cl_2 and Me_2CO to give **15** (yellow solid, 0.8 mg), **19** (yellow solid, 1.3 mg), and **14** (30.4 mg) as a pale yellow solid. Fraction TA7.8 (0.471 g) was crystallized (100% Me_2CO) to yield a yellow solid of **15** (15.4 mg). The filtrate (0.455 g) was purified by CC and eluted with a step gradient of CH_2Cl_2 and Me_2CO to give **14** (29.5 mg) as a pale yellow solid. Fractions TA7.9 and TA7.10 were combined (0.220 g) and purified by CC (20%-25% Me_2CO-hexanes) to give **14** (pale yellow solid, 18.5 mg) and **15** (yellow solid, 21.1 mg). Subfraction TA7.11 (0.173 g) was separated on CC (35%-40% EtOAc-hexanes) and CC (50%-60% EtOAc-hexanes) to yield **23** (pale yellow solid, 5.0 mg). Fraction TA8 (brown solid, 0.920 g) was subjected to CC (40% EtOAc-hexanes) to give seven fractions. Fraction TA8.2 (79.2 mg) was purified by CC (40% EtOAc-hexanes) to give **14** (pale yellow solid, 33.0 mg) and **21** (orange solid, 2.7 mg). Fraction TA8.4 (180.1 mg) was crystallized (10% Me_2CO-CH_2Cl_2) to give **20** (17.0 mg) as a pale yellow solid. Fractions TA9 and TA10 were combined (brown solid, 3.335 g) and subjected to CC (30%-75% Me_2CO-hexanes) and recrystallized (50% Me_2CO-CH_2Cl_2) to afford **22** (7.8 mg) as a pale yellow solid (as shown in figure 3-7).

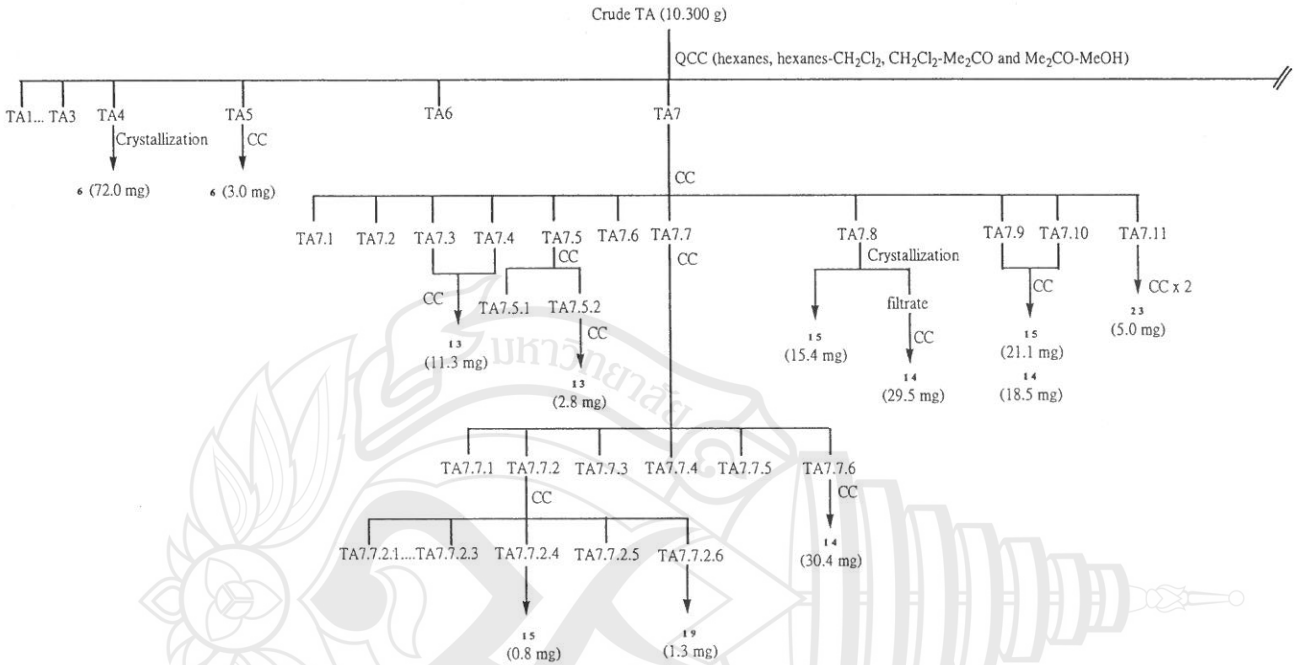


Figure 3-7 Isolation of Pure Compounds from Crude TA

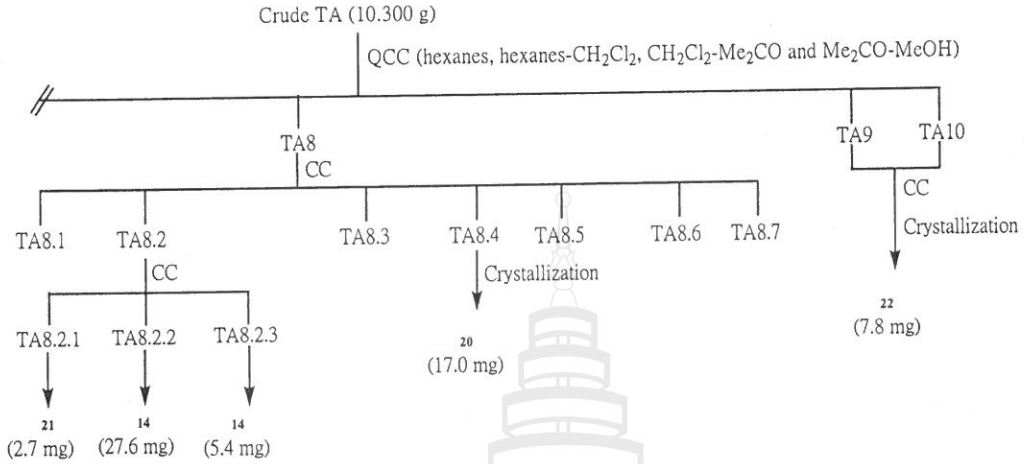


Figure 3-7 (continued)

3.5 Antibacterial Activity Assay

Broth microdilution method (Clinical and Laboratory Standards Institute [CLSI.], 2002) was used to screen and determine minimum inhibition concentrations (MICs) of crude extracts and pure compounds.

3.5.1 Screening of Crude Extracts and Pure Compounds

Test samples were dissolved in DMSO and mixed with melted MHB in microtiter plates. Add 50 µL of inoculum suspensions in each well. Final concentration of the test samples was 1,000 µg/mL (crude extracts) and 200 µg/mL (pure compounds). The inoculated plate were incubated at 35-37 °C for 16-18 h. Drop 0.18% resazurin 10 µL in microtiter plate and incubated in 35-37 °C for 2-3 h. The blue color showed that the sample can inhibit bacterial growth whereas the pink color indicated that the sample can't inhibit bacterial growth. This was performed in triplicate for each sample. Vancomycin and gentamicin were used as positive control drugs.

3.5.2 Determination of Minimum Inhibition Concentrations (MICs) of Crude Extracts and Pure Compounds

Test samples were dissolved in DMSO. Serial 2-fold dilutions of the test samples were mixed with melted MHB in microtiter plates. Final concentration of the test crude sample and pure compound in broth ranged from 1280–2.5 µg/mL and 128-0.25 µg/mL, respectively. Add 50 µL of inoculum suspensions in each well (final concentration of $1x10^4$ CFU/well). The inoculated plates were incubated at 35-37 °C for 16-18 h. Drop 0.18% resazurin 10 µL in microtiter plate and incubated in 35-37 °C for 2-3 h. The blue color showed that the sample can inhibit bacterial growth, while the pink color indicated that the samples can't inhibit bacterial growth. MICs were recorded by reading the lowest concentration that inhibited visible growth. The tests were performed at least in triplicate. Vancomycin and gentamicin were used as positive control drugs.

3.6 Anticancer Activity Assay

KB (Human epidermoid carcinoma of cavity, ATCC CCL-17), MCF7 (Human breast adenocarcinoma, ATCC HTB-22) and NCI-H187 (Human small cell lung carcinoma, ATCC CRL-5804) were determined by resazurin microplate assay (REMA) which was a modified method of fluorescent dye for the mammalian cell cytotoxicity according to Brien et al. (2000). Ellipticine and doxorubicin were used as positive control. DMSO and sterile distilled water was used as negative control. Briefly, cells at a logarithmic growth phase were harvested and diluted to 10^5 cells/ml in fresh medium and gently mixed. Test compounds were diluted in culture medium at a ration of 1:2 giving 8 concentrations. Five microlitres of test sample and 45 microlitres of cells were put into 384-well microtiter plates in total volume of 50 µl/well. Plates were incubated at 37 °C, 5% CO_2 for 72 h for KB and MCF7, and 5 days for NCI-H187. After incubation period, 12.5 microlitres of resazurin solution was added to each well and the plates were incubated at 37 °C for 4 h. The plates were then processed for optical density absorbance analysis using Victor 3 Microplate reader at dual wavelengths of 530 and 590 nm.

3.7 DPPH Radical Scavenging Assay

The potential antioxidant activities of the crude extracts and pure compounds isolated from *C. alata* (flowers, leaves, roots, stems and twigs) was assessed on the basis of scavenging activity of the stable (DPPH) free radical. The DPPH assay is one of the methods used for evaluation of antioxidative activity. The following assay procedure was modified from those described in previous report (Deachathai et al., 2006). The test solution in absolute ethanol (50 μL) was mixed with 0.05 mM DPPH solution in absolute ethanol (3 mL). The absorbance (Abs) was then measured at 517 nm on spectrophotometer. BHT and ascorbic acid were used as a positive control. The measurements were performed at least in triplicate. The result expressed as percentage inhibition. The concentration of the sample at 50% inhibition (IC_{50}) was obtained by linear regression analysis of dose-response curve, which was plotted between % inhibition and concentration.

$$\% \text{ inhibition} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

3.7.1 Screening on the Free Radical Scavenging Activity of Crude Extracts and Pure Compounds

The crude material was dissolved in absolute ethanol to prepare the solution with concentration of 6.1 mg/mL. The solution of each sample (50 μL) was mixed with 0.05 mM DPPH ethanolic solution (3 mL) in a cuvette to give the solution with the final concentration of 100 μg/mL. The pure compound was dissolved in absolute ethanol to prepare the solution with concentration of 0.61 mM. The solution of each sample (50 μL) was mixed with 0.05 mM DPPH ethanolic solution (3 mL) in a cuvette to give the solution with the final concentration of 10 μM. The trapping effect was assessed by measuring the absorbance change of the solution at 517 nm against 0.05 mM DPPH ethanolic solution after 15, 30, 45 and 60 min. Ascorbic acid and BHT were used as a positive control. The measurements were performed at least in triplicate. The degree of loss of color implied the activity.

3.7.2 Determination of 50% Inhibition Concentration (IC_{50}) of Crude Extracts and Pure Compounds

The solution of DPPH (0.05 mM, 3 mL) was mixed with the sample at various concentrations of a crude extract in mg/mL and a pure compound in mM. The absorbances were measured at 517 nm for 30 min. The concentration that needed to decrease % inhibition of DPPH solution to 50% inhibition (IC_{50}) was obtained by linear regression analysis of dose-response curve. The measurements were performed at least in triplicate.

CHAPTER 4
RESULTS AND DISCUSSION

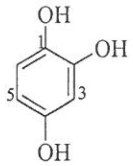
4.1 Isolated Compounds from *Cassia alata*

Investigation of the chemical constituents of the extracts obtained from the flowers, leaves, roots, stems and twigs of *C. alata* resulted in the isolation of 23 compounds as shown in table 4-1. Isolation and purification of the dichloromethane and acetone extracts of the flowers gave five compounds (**1-5**) and two compounds (**5** and **6**), respectively. The dichloromethane and acetone extracts of the leaves gave one compound (**5**) and four compounds (**5-8**), respectively. Twelve compounds (**3**, **4**, **6**, and **8-16**) were obtained from acetone extract of the roots. The dichloromethane and acetone extracts of the stems gave three compounds (**4**, **9**, and **17**) and five compounds (**4**, **6**, **9**, and **18**), respectively. Three compounds (**4**, **6**, and **9**) were isolated from the dichloromethane extract and eleven compounds (**4**, **6**, **9**, **13**, **14**, **15**, **19**, **20**, **21**, **22**, and **23**) were obtained from the acetone extract of twigs (as shown in table 4-1). Sixteen compounds of them (**1**, **2**, **4**, **8**, **9**, **11-19**, **21**, and **22**) were reported for the first time as metabolites of *C. alata*.

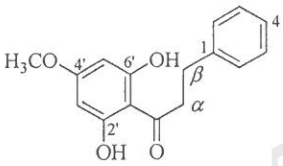
Table 4-1 Isolated Compounds from *Cassia alata*

Part	Extract	Isolated compound
flowers	dichloromethane	**1, 2, 3, 4, 5**
	acetone	**5, 6**
leaves	dichloromethane	**5**
	acetone	**5, 6, 7, 8**
roots	acetone extract	**3, 4, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16**
stems	dichloromethane	**4, 9, 17**
	acetone	**4, 6, 9, 18**
twigs	dichloromethane	**4, 6, 9**
	acetone	**4, 6, 9, 13, 14, 15, 19, 20, 21, 22, 23**

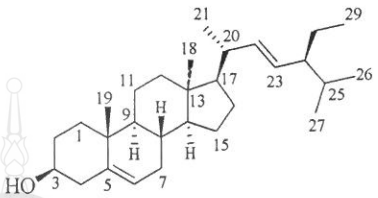
Structural Elucidation of Isolated Compounds from *Cassia alata*



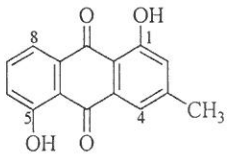
Compound 1



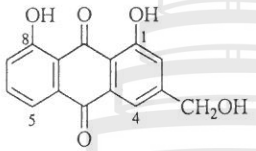
Compound 2



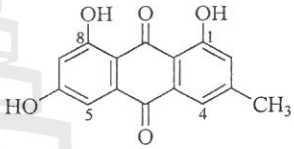
Compound 3



Compound 4



Compound 5



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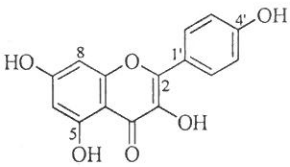

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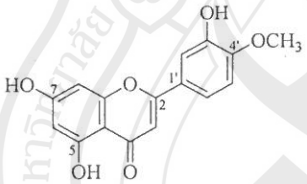
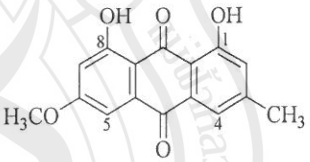


Compound 8



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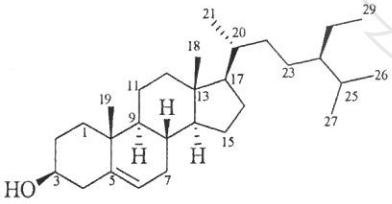

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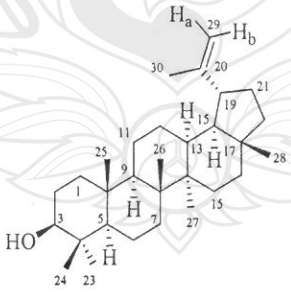

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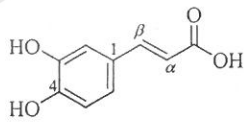

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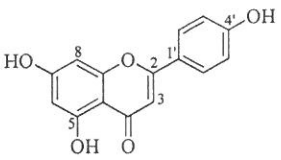
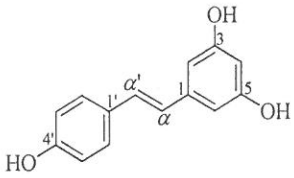


Compound 13



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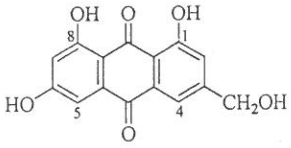

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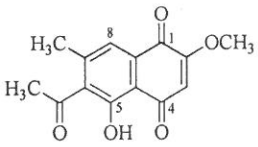
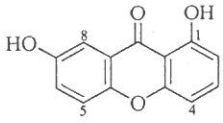
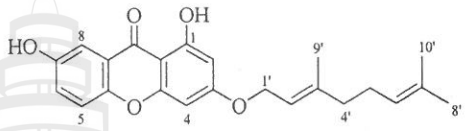


Compound 16



Compound 17



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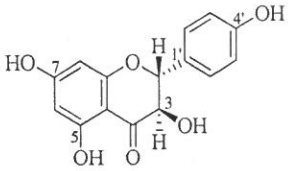

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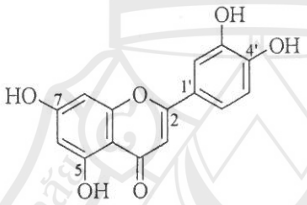
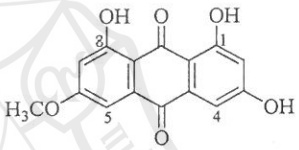
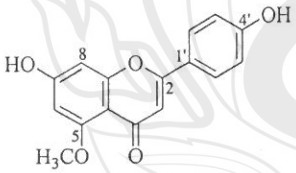


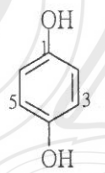
Compound 20



Compound 21



Compound 22



Compound 23

Compound **1**, 1,2,4-trihydroxybenzene or hydroxyquinol, was obtained as a yellow solid. The UV spectrum (in MeOH) exhibited maximum absorptions (log ε) at 211.0 (3.91), 254.7 (3.83) and 290.3 (3.72) nm. The IR (KBr) spectrum showed the stretching of hydroxyl group (3273 cm^{-1}). The ^1H NMR spectrum (table 4-2) showed the resonances of ABX pattern of aromatic protons at δ7.45 (1H, *d*, J = 9.3 Hz, H-6), 8.51 (1H,*dd*, J = 2.7, 9.3 Hz, H-5) and8.92 (1H, *d*, J = 2.7 Hz, H-3), respectively. The elucidated structure was confirmed by HMBC correlations of H-3 to C-1, C-2, C-4, C-5, H-5 to C-1, C-3, C-4 and H-6 toC-1, C-2, C-4.

Table 4-2 The NMR Spectral Data of Compound **1**

Position	1 (300 MHz in acetone-d_6)		
	δ_H (*mult.*, J_{Hz})	δ_C	HMBC
1	-	140.0	-
2	-	134.8	-
3	8.92 (1H, *d*, 2.7)	123.0	C-1, C-2, C-4, C-5
4	-	159.7	-
5	8.51 (1H, *dd*, 9.3, 2.7)	132.1	C-1, C-3, C-4
6	7.45 (1H, *d*, 9.3)	122.3	C-1, C-2, C-4

Compound **2**, 2′,6′-dihydroxy-4′-methoxydihydrochalcone, was obtained as a brown solid. The UV spectrum in MeOH exhibited maximum absorptions (log ε) at 208.5 (4.30), 226.7 (4.18) and 285.0 (4.25) nm. The IR (KBr) spectrum showed the stretching of hydroxyl (3261 cm^{-1}) and carbonyl group (1645 cm^{-1}) groups. The ^1H NMR spectrum (table 4-3) showed the signals of two equivalent aromatic protons at δ5.99 (2H, *s*, H-3′,5′), a methoxy group at δ3.79 (3H, *s*, 4′-OCH_3), a set of monosubstituted benzene ring at δ 7.25 (2H, *m*, H-2,6), 7.24 (2H, *m*,H-3,5) and 7.18 (1H, *m*, H-4). Furthermore, the ^1H NMR showed two coupled deshielded methylene groups at δ3.41 (2H, *t*, J = 4.8 Hz, H_2-α) and 2.98 (2H, *t*, J = 4.8 Hz, H_2-β) charactreristic of a dihydrochalcone derivertive. The HMBC correlations of H_2-β to C-1, C-2,6; H_2-α to C-1 and H-2,6 to C-β, C-1 indicated the position of monosubstituted benzene. The correlations of equivalent aromatic protons H-3′,5′ to

C-1′, C-2′,6′, C=O confirmed the position of substituted aromatic ring and the correlation of 4′-OCH_3 to C-4′ indicated the position of methoxy group at C-4′. (Masuoka, Ono, Ito & Nohara, 1997).

Table 4-3 The NMR Spectral Data of Compound **2**

Position	2 *			Masuoka et al., 1997 **	
	δ_H (*mult.*, J_{Hz})	δ_C (DEPT)	HMBC	δ_H (*mult.*, J_{Hz})	δ_C
1	-	143.0 (C)	-	-	143.1
2,6	7.25 (2H, *m*)	129.3/129.4 (CH)	C-1, C-2,6, C-3,5, C-4, C-β	*ca.* 7.27 (2H)	129.4/129.5
3,5	7.24 (2H, *m*)	129.4/129.3 (CH)	C-2,6, C-3,5, C-4	*ca.* 7.27 (2H)	129.5/129.4
4	7.18 (1H, *m*)	126.8 (CH)	-	7.18 (1H, *br t*, 2.2)	126.9
1′	-	105.8 (C)	-	-	106.0
2′,6′	-	165.4 (C)	-	-	165.6
3′,5′	5.99 (2H, *s*)	94.5 (CH)	C-1′, C-2′,6′, C-3′,5′,C-4′, C=O	6.00 (2H, *s*)	94.4
4′	-	167.0 (C)	-	-	167.5
α	3.41 (2H, *t*, 4.8)	46.6 (CH_2)	C-1, C-β, C=O	3.40 (2H, *m*)	47.0
β	2.98 (2H, *t*, 4.8)	31.4 (CH_2)	C-1, C-2,6, C-α, C=O	3.00 (2H, *m*)	32.0
C=O	-	205.7 (C)	-	-	206.4
4′-OCH_3	3.79 (3H, *s*)	55.9 (CH_3)	C-4′	3.79 (3H, *s*)	55.8

Note. *300 MHz in acetone-d_6
**400 MHz in acetone-d_6

Compound **3**, 5,22-stigmastadien-3β-ol or stigmasterol, was obtained as a white solid. The ^{1}H NMR spectrum contained an oxymethine proton signal at δ 3.46 (*m*, H-3), three olefinic protons at δ 5.28 (*d*, J = 4.8 Hz, H-6), 5.08 (*m*, H-22) and 4.94 (*m*, H-23) and six methyl groups at δ 1.02 (*br s*, 21-CH_3), 1.02 (*s*, 19-CH_3), 0.74×2 (*brs*, 27-CH_3, 29-CH_3), 0.74 (*s*, 26-CH_3) and 0.62 (*s*, 18-CH_3). The ^{1}H and ^{13}C NMR data were corresponded to the previous reported data (Forgo & Kover, 2004).

Compound **4** (1,5-dihydroxy-3-methylanthraquinone, ziganein) was obtained as a yellowish red solid, m.p. 226-228 °C, (227-228 °C, Lee, C.-L., Lee, P.-H. & Kuo, 2001). The UV spectrum in MeOH exhibited the maximum absorptions (log ε) at 223.2 (4.12), 264.6 (3.84), 286.0 (3.81) and 433.1 (3.61) nm. The IR (KBr) spectrum showed the stretching of hydroxy (3359 cm^{-1}) and carbonyl (1629 cm^{-1}) groups. The ^1H NMR spectral data (table 4-4) exhibited signals of two chelated hydroxyl protons at δ 12.02 (*s*, 1-OH) and 12.13 (*s*, 5-OH), and a methyl proton at δ 2.47(*s*, 3-CH_3). The spectrum further showed the resonances of *meta* protons H-2 and H-4 at δ 7.11 (*d*, J = 1.0 Hz) and 7.66 (*d*, J = 1.0 Hz), respectively. The remaining resonances were a *doublet of doublet* signal at δ 7.30 (J = 8.0, 1.0 Hz), a *triplet* signal at δ 7.69 (J = 8.0 Hz) and a *doublet of doublet* signal at δ 7.83 (J = 8.0, 1.0 Hz) which were assigned for the resonances of ABM system of H-6, H-7 and H-8, respectively. These results suggested that FDC9 was an anthraquinone skeleton. Two quaternary signals at δ 182.0 and 181.9 suggested the carbonyl carbons of **4** to be attributable to ketone form. Correlations of 1-OH to C-1, C-2, C-3 and C-9a and 5-OH to C-5, C-6, C-7, C-10 and C-10a supported two chelated hydroxy groups were at C-1 and C-5, respectively. In addition, the correlations of 3-CH_3 to C-2, C-3 and C-4 supported the position of CH_3 at C-3 (Lim, 1999).

Table 4-4 The NMR Spectral Data of Compound **4**

Position	4 *			Lim, 1999 **	
	δ_H (*mult.*, J_{Hz})	δ_C (DEPT)	HMBC	δ_H (*mult.*, J_{Hz})	δ_C
1	-	162.8 (C)	-	-	162.68
2	7.11 (1H, *d*, 1.0)	124.3 (CH)	C-1, C-3, C-4, C-9a	7.09 (1H, *d*, 1.4)	124.32
3	-	149.3 (C)	-	-	149.30
4	7.66 (1H, *d*, 1.0)	121.3 (CH)	C-2, C-3, C-4a, C-9a, C-10	7.65 (1H, *m*)	121.31
4a	-	133.2 (C)	-	-	133.25
5	-	162.7 (C)	-	-	162.38
6	7.30 (1H, *dd*, 8.0, 1.0)	124.5 (CH)	C-5, C-8, C-10a	7.29 (1H, *dd*, 7.53, 1.20)	124.51
7	7.69 (1H, *t*, 8.0)	136.9 (CH)	C-5, C-8	7.69 (1H, *d*, 7.53)	136.90
8	7.83 (1H, *dd*, 8.0, 1.0)	119.9 (CH)	C-6, C-9	7.83 (1H, *dd*, 7.53, 1.20)	119.88
8a	-	133.5 (C)	-	-	133.61

9	-	182.0 (C)	-	-	192.49
9a	-	113.9 (C)	-	-	113.70
10	-	181.9 (C)	-	-	181.93
10a	-	115.9 (C)	-	-	115.84
1-OH	12.02 (1H, *s*)	-	C-1, C-2, C-3, C-9a	11.99 (1H, *s*)	-
5-OH	12.13 (1H, *s*)	-	C-5, C-6, C-7, C-10, C-10a	12.11 (1H, *s*)	-
3-CH_3	2.47 (3H, *s*)	22.3 (CH_3)	C-2, C-3, C-4	2.46 (3H, *s*)	21.21

Note. *400 MHz in acetone-d_6

**300 MHz in $CDCl_3$

Compound **5** (1,8-dihydroxy-3-(hydroxymethyl)anthraquinone, aloe-emodin) was obtained as an orange solid, m.p. 220-221 °C, (221-222 °C, Gavit & Laddha, 2012). The UV in MeOH spectra [λ_{max}(log ε) : 225.3 (4.56), 255.4(4.29),286.4 (3.96) and 428.5 (3.99) nm] indicated an anthraquinone nucleus. The IR (KBr) absorption bands at 3419 and 1626 cm^{-1} suggested the presence of hydroxyl and carbonyl groups, respectively. The ^{1}H NMR spectral data (table 4-5) showed signals of two chelated hydroxyl protons at δ 12.09 (*s*, 1-OH) and 12.04 (*s*, 8-OH), hydroxymethyl protons at δ4.72 (*br d*, 3-C$\underline{H_2}$OH) and 5.01 (*br t*, 3-CH_2O$\underline{H}$). The resonances of *meta* protons H-2 and H-4 were observed at δ7.34 (*s*, 1H) and 7.78 (*s*, 1H), respectively. The remaining resonances were assigned to be aromatic protons H-5 (δ7.80, *d*, J = 8.1 Hz), H-6 (δ7.67, *t*, J = 8.1 Hz) and H-7 (δ7.28, *d*, J = 8.1 Hz), respectively. The ^{13}C NMR spectrum exhibited a hydroxymethylene at δ 64.0 and two carbonyl carbons at δ 181.5 and 194.0. The HMBC correlations of 3-C$\underline{H_2}$OH to C-2, C-3 and C-4 indicated the position of hydroxymethyl group at C-3. The position of *meta* aromatic protons H-2 and H-4 were confirmed by the correlations of H-2 to C-1, 3-$\underline{C}H_2$OH, C-4, C-9a and H-4 to 3-$\underline{C}H_2$OH, C-4a, C-9a, C-10. The correlations of H-5 to C-7, C-8a, C-10; H-6 to C-8, C-10a; H-7 to C-5, C-8a confirmed the position of aromatic protons H-5, H-6 and H-7, respectively (Kametani et al., 2007).

Table 4-5 The NMR Spectral Data of Compound **5**

Position	5 *			Kametani et al., 2007 **	
	δ_H (*mult.*, J_{Hz})	δ_C	HMBC	δ_H (*mult.*, J_{Hz})	δ_C
1	-	162.9	-	-	161.5
2	7.34 (1H, *s*)	121.6	C-1, 3-$\underline{C}H_2OH$, C-4, C-9a	7.30 (1H, *d*, 1.7)	120.6
3	-	151.7	-	-	153.6
4	7.78 (1H, *s*)	118.1	3-$\underline{C}H_2OH$, C-4a, C-9a, C-10	7.71 (1H, *d*, 1.7)	117.0
4a	-	133.8	-	-	133.1
5	7.80 (1H, *d*, 8.1)	120.0	C-7, C-8a, C-10	7.73 (1H, *dd*, 7.6, 1.2)	119.2
6	7.67 (1H, *t*, 8.1)	137.0	C-8, C-10a	7.81 (1H, *dd*, 8.3, 7.6)	137.2
7	7.28 (1H, *d*, 8.1)	125.0	C-5, C-8a	7.38 (1H, *dd*, 8.3, 1.2)	124.2
8	-	163.0	-	-	161.2
8a	-	118.2	-	-	116.8
9	-	194.0	-	-	191.5
9a	-	112.0	-	-	114.4
10	-	181.5	-	-	181.4
10a	-	134.0	-	-	133.3
1-OH	12.09 (1H, *s*)	-	C-1, C-2, C-9a	11.90 (1H, *br s*)	-
3-$\underline{C}H_2OH$	4.72 (2H, *br d*)	64.0	C-2, C-3, C-4	4.63 (2H, *br s*)	62.0
3-$CH_2O\underline{H}$	5.01 (1H, *br t*)	-	-	5.52 (1H, *br s*)	-
8-OH	12.04 (1H, *s*)	-	C-7, C-8, C-8a	11.96 (1H, *br s*)	-

Note. *300 MHz in $CDCl_3$+DMSO-d_6
**500 MHz in DMSO-d_6

Compound **6** (1,6,8-trihydroxy-3-methylanthraquinone, emodin) was obtained as an orange solid, m.p. 252-254 °C, (254-256 °C, Zhou et al., 2006). The UV spectrum (in MeOH) exhibited maximum absorptions (log ε) at 220.1 (4.12), 253.1 (3.89), 289.7 (3.87) and 438.5 (3.60) nm. The IR (KBr) spectrum showed the stretching of hydroxyl (3359 cm^{-1}) and carbonyl (1627 cm^{-1}) groups. The ^1H NMR spectral data (table 4-6) showed signals of two chelated hydroxyl protons at δ12.09 (*br s*, 1-OH) and 12.20 (*br s*, 8-OH) and a methyl proton at δ2.47 (*s*, 3-CH_3). The spectrum further showed the resonances of *meta* coupled protons H-5 and H-7 at δ7.26 (*d*, J = 2.4 Hz) and 6.66 (*d*, J = 2.4 Hz), respectively. The remaining resonances

were two *broad singlet* signals at δ 7.14 and7.57, which were assigned for the resonances of *meta* H-2 and H-4, respectively. The HMBC correlations of3-CH_3 to C-2, C-3 and C-4 indicated the position of methyl group at C-3. The position of aromatic proton H-2 was confirmed by the correlations of H-2 to C-1, C-4 and C-9a and H-4 was confirmed by the correlations of H-4 to C-2, C-9a and C-10. The correlations of H-5 to C-7, C-8a and C-10 and H-7 to C-5, C-8 and C-8a confirmed the position of aromatic protons H-5 and H-7, respectively (Chu, Sun & Liu, 2005).

Table 4-6 The NMR Spectral Data of Compound **6**

Position	6 *			Chu et al., 2005 **	
	δ_H (*mult.*, J_{Hz})	δ_C (DEPT)	HMBC	δ_H (*mult.*, J_{Hz})	δ_C
1	-	167.0 (C)	-	-	164.4
2	7.14 (1H, *br s*)	125.3 (CH)	C-1, C-4, C-9a	7.07 (1H, *s*)	120.4
3	-	150.0 (C)	-	-	148.2
4	7.57 (1H, *br s*)	121.8 (CH)	C-2, C-9a, C-10	7.42 (1H, *s*)	124.0
4a	-	136.9 (C)	-	-	132.7
5	7.26 (1H, *d*, 2.4)	110.2 (CH)	C-7, C-8a, C-10	7.11 (1H, *s*)	108.8
6	-	163.4 (C)	-	-	161.4
7	6.66 (1H, *d*, 2.4)	109.1 (CH)	C-5, C-8, C-8a	6.56 (1H, *s*)	107.9
8	-	166.5 (C)	-	-	165.6
8a	-	110.1 (C)	-	-	108.7
9	-	189.3 (C)	-	-	189.6
9a	-	113.7 (C)	-	-	113.3
10	-	182.8 (C)	-	-	181.2
10a	-	136.9 (C)	-	-	135.0
1-OH	12.09 (1H, *br s*)	-	-	11.96 (1H, *s*)	-
8-OH	12.20 (1H, *br s*)	-	-	12.04 (1H, *s*)	-
3-CH_3	2.47 (3H, *s*)	22.3 (CH_3)	C2, C-3, C-4	2.38 (3H, *s*)	21.5

Note. *400 MHz in acetone-d_6

**100 MHz in DMSO-d_6

Compound 7 (5,7,4′-trihydroxyflavonol, kaempferol) was obtained as a yellow solid, m.p. 177-179 °C, (178-180 °C, Lee et al., 2007). This compound exhibited UV absorption bands in MeOH (log ε) at 203.3 (4.52), 266.2 (4.28) and 365.3 (4.36) nm, a characteristic of a flavone nucleus and IR (KBr) absorption bands at 3331 and 1660 cm^{-1}. The ^1H NMR spectrum (table 4-7) showed the resonances of a hydrogen bonded hydroxy proton at δ12.14 (*s*, 5-OH), three free hydroxy groups at δ7.90 (*br s*, 3-OH), 10.17 (*br s*, 7-OH), and 9.53 (*s*, 4′-OH). Two *doublet* resonances (J = 1.5 Hz) at δ 6.27 (1H) and 6.42 (1H) were in agreement with the *meta*-coupling of aromatic protons H-6 and H-8, respectively. The remaining ^1H signals showed the resonances of an AA′BB′ pattern at δ 8.09 (2H, *d*, J = 9.9 Hz) and 6.95 (2H, *d*, J = 9.9 Hz) implied the presence of H-2′,6′ and H-3′,5′, respectively (Lee et al., 2007).

Table 4-7 The NMR Spectral Data of Compound 7

Position	7 *			Lee et al., 2007 **	
	δ_H (*mult.*, J_{Hz})	δ_C (DEPT)	HMBC	δ_H (*mult.*, J_{Hz})	δ_C
2	-	146.2 (C)	-	-	147.8
3	-	135.5 (C)	-	-	137.1
4	-	175.5 (C)	-	-	177.1
4a	-	103.2 (C)	-	-	104.4
5	-	160.9 (C)	-	-	162.3
6	6.27 (1H, *d*, 1.5)	98.6 (CH)	C-4a, C-5, C-7, C-8	6.16 (*d*, 2.0)	99.2
7	-	164.0 (C)	-	-	165.3
8	6.42 (1H, *d*, 1.5)	93.8 (CH)	C-4a, C-6, C-7, C-8a	6.36 (*d*, 2.0)	94.4
8a	-	156.6 (C)	-	-	158.1
1′	-	122.0 (C)	-	-	123.6
2′,6′	8.09 (2H, *d*, 9.9)	129.4 (CH)	C-2, C-2′,6′, C-3′, C-4′	8.06 (*d*, 9.2)	130.5
3′,5′	6.95 (2H, *d*, 9.9)	115.5 (CH)	C-1′, C-3′,5′, C-4′	6.89 (*d*, 9.2)	116.2
4′	-	159.1 (C)	-	-	160.3
3-OH	7.90 (1H, *br s*)	-	C-2, C-3, C-4	-	-
5-OH	12.14 (1H, *s*)	-	C-4a, C-5, C-6	-	-
7-OH	10.17 (1H, *br s*)	-	C-6, C-7, C-8	-	-
4′-OH	9.53 (1H, *s*)	-	C-3′, C-4′, C-5′	-	-

Note. *300 MHz in acetone-d_6

Compound **8** (5,7,3′-trihydroxy-4′-methoxyflavone, diosmetin) was obtained as a yellow solid. The ^{1}H NMR spectrum (table 4-8) showed the characteristic resonances of a flavone proton at δ 6.70 (*s*, H-3), a hydrogen-bonded hydroxyl proton at δ 13.02 (*s*, 5-OH) and a *meta* coupled protons H-6 and H-8 at δ 6.26 (1H, *d*, *J* = 2.1 Hz) and 6.55 (1H, *d*, *J* = 2.1 Hz). The spectrum further exhibited the resonances of ABM pattern of H-2′ (δ 7.64, *d*, *J* = 2.4 Hz), H-5′ (δ7.01, *d*, *J* = 8.1 Hz) and H-6′ (δ7.61, *dd*, *J* = 2.4, 8.1 Hz). The presence of a methoxy group was shown in the spectrum, of which the signal at δ 4.00 (3H, *s*, 4′-OCH_3). The HMBC correlations of 4′-OCH_3 to C-4′ indicated the position of methoxy group at C-4′. The proof of vinylic proton H-3 was obtained from the results of ^2J cross peaks of H-3 to C-2 (δ166.5) and ^3J cross peak of H-3 to C-4a (δ106.0) (Ahn et al., 2011).

Table 4-8 The NMR Spectral Data of Compound **8**

Position	**8** *			Ahn et al., 2011 **	
	δ_H (*mult.*, J_{Hz})	δ_C (DEPT)	HMBC	δ_H (*mult.*, J_{Hz})	δ_C
2	-	166.5 (C)	-	-	165.9
3	6.70 (1H, *s*)	104.9 (CH)	C-2, C-4a	6.54 (1H, *s*)	104.4
4	-	186.6 (C)	-	-	183.8
4a	-	106.0 (C)	-	-	105.3
5	-	165.0 (C)	-	-	163.2
6	6.26 (1H, *d*, 2.1)	100.3 (CH)	C-8	6.19 (1H, *d*, 2.0)	100.2
7	-	N/D	-	-	166.2
8	6.55 (1H, *d*, 2.1)	95.3 (CH)	-	6.41 (1H, *d*, 2.0)	95.1
8a	-	N/D	-	-	159.4
1′	-	N/D	-	-	125.0
2′	7.64 (1H, *d*, 2.4)	111.1 (CH)	C-2, C-3′, C-4′	7.35 (1H, *d*, 2.0)	113.9
3′	-	152.0 (C)	-	-	148.2
4′	-	149.0 (C)	-	-	152.6
5′	7.01 (1H, *d*, 8.1)	116.9 (CH)	-	7.04 (1H, *d*, 8.8)	112.7
6′	7.61 (1H, *dd*, 8.1, 2.4)	121.9 (CH)	C-2, C-4′	7.45 (1H, *dd*, 8.8, 2.0)	120.0
5-OH	13.02 (1H, *s*)	-	C-4a, C-5, C-6	-	-
4′-OCH_3	4.00 (3H, *s*)	57.1 (CH_3)	C-4′	3.92 (3H, *s*)	56.5

Compound **9** (1,8-dihydroxy-6-methoxy-3-methylanthraquinone, physcion) was isolated as a yellow solid, m.p. 206-208°C, (207-209 °C, Zhou et al., 2006). The UV spectrum (in MeOH) exhibited maximum absorption bands and log ε at 223.2 (4.23), 264.8 (3.95), 286.2 (3.92) and 433.3 (3.74) nm. The IR (KBr) spectrum showed the absorption bands of stretching of hydroxyl (3357 cm^{-1}) and carbonyl (1631 cm^{-1}) groups. The ^1H NMR spectral data (table 4-9) showed two sharp *singlet* signals of two chelated phenolic hydroxyl protons at δ 12.05 (*s*, 1-OH) and 12.24 (*s*, 8-OH). The presence of a methoxy proton and a methyl proton were shown in the spectrum, of which the *singlet* signals at δ3.94 (*s*, 6-OCH_3) and 2.38 (*s*, 3-CH_3), respectively. Two sets of resonances characteristic of *meta* protons were shown as two *doublets* at δ7.01 and 7.55 (H-2 and H-4, J = 1.2 Hz each) and two *doublets* at δ 7.29 and 6.61 (H-5 and H-7, J = 2.4 Hz each). The HMBC correlation of 6-OCH_3 to C-6 suggested the position of OCH_3 at C-6, whereas 3-CH_3 correlated to C-2, C-3 and C-4 indicating the methyl group at C-3 (Chu et al., 2005).

Table 4-9 The NMR Spectral Data of Compound **9**

Position	9 *			Chu et al., 2005 **	
	δ_H (*mult.*, J_{Hz})	δ_C (DEPT)	HMBC	δ_H (*mult.*, J_{Hz})	δ_C
1	-	162.5 (C)	-	-	164.7
2	7.01 (1H, *d*, 1.2)	124.5 (CH)	C-1, C-4, C-9a	7.09 (1H, *s*)	120.7
3	-	148.5 (C)	-	-	148.0
4	7.55 (1H, *d*, 1.2)	121.3 (CH)	C-2, C-9a, C-10	7.64 (1H, *s*)	124.0
4a	-	133.2 (C)	-	-	132.7
5	7.29 (1H, *d*, 2.4)	108.2 (CH)	C-7, C-8a, C-10	7.32 (1H, *s*)	107.8
6	-	166.6 (C)	-	-	161.9
7	6.61 (1H, *d*, 2.4)	106.8 (CH)	C-5, C-8, C-8a	6.74 (1H, *s*)	106.1
8	-	165.2 (C)	-	-	166.1
8a	-	110.3 (C)	-	-	110.2
9	-	190.8 (C)	-	-	190.2
9a	-	113.7 (C)	-	-	113.1
10	-	182.0 (C)	-	-	181.6
10a	-	135.3 (C)	-	-	134.7
1-OH	12.05 (1H, *s*)	-	C-1, C-2, C-9a	12.06 (1H, *s*)	-
8-OH	12.24 (1H, *s*)	-	C-7, C-8, C-8a	12.19 (1H, *s*)	-

6-OCH_3	3.94 (3H, *s*)	56.1 (OCH_3)	C-6	3.92 (3H, *s*)	55.7
3-CH_3	2.38 (3H, *s*)	22.2 (CH_3)	C-2, C-3, C-4	2.42 (3H, *s*)	21.7

Note. *400 MHz in acetone-d_6

**400 MHz in DMSO-d_6 + $CDCl_3$

Compound **10** (stigmast-5-en-3β-ol, β-sitosterol) was obtained as a white solid, The ^{1}H NMR spectrum showed the presence of an olefinic proton at δ 5.36 (1H, *m*, H-6) and an oxymethine proton at δ 3.53 (1H, *m*, H-3). The signals of six methyl groups were shown at δ0.63 (*s*, H-18), 0.81 (*d*, J = 6.5 Hz, H-27), 0.84 (*d*, J = 6.5 Hz, H-26), 0.85 (*t*, J = 8.0 Hz, H-29), 0.92 (*d*, J = 6.5 Hz, H-21) and 1.01 (*s*, H-19). ^{1}H NMR spectral data were corresponded to the previously reported values (Nguyen et al., 2004).

Compound **11** (lup-20(29)-en-3β-ol, lupeol) was obtained as a white solid, The ^{1}H NMR spectrum exhibited the characteristic signal of a terminal olifinic methylene protons at δ 4.68 and 4.56 (1H each, *d*, J = 2.4 Hz) for H_b-29 and H_a-29, respectively. The ^{1}H NMR spectrum also showed the resonances of an oxymethine proton (δ 3.39, *dd*, J = 5.7 and 1.5 Hz, H-3) and seven methyl groups [(δ 0.96 (*s*, H-23), 0.84 (*s*, H-24), 0.82 (*s*, H-25), 1.03 (*s*, H-26), 0.93 (*s*, H-27), 0.78 (*s*, H-28) and 1.68 (*s*, H-30)] (Imam, Azhar, Hasan, Ali & Ahmed, 2007).

Compound **12** (3,4-dihydroxy cinnamic acid, caffeic acid) was obtained as a brown solid. The ^{1}H NMR spectrum (table 4-10) exhibited the signals of 1,3,4-trisubstituted benzene derivative at δ7.15 (1H, *d*, J = 2.1 Hz, H-2),6.86 (1H, *d*, J = 8.1 Hz, H-5) and7.03 (1H, *d*, J = 2.1, 8.1 Hz, H-6). The *trans* stereochemistry was deduced from the peaks at δ7.53 (1H, *d*, J = 15.9 Hz, H-β) and 6.28 (1H, *d*, J = 15.9 Hz, H-α). The ^{13}C NMR spectrum showed a signal of C=O at δ168.0, two signals ofoxygenated carbons at δ 147.2 (C-3) and 149.3 (C-4), a quaternary carbon at δ128.1 (C-1), three methine carbons at δ115.7 (C-2), 116.9 (C-5) and 122.9 (C-6), and two olefinic carbons at δ146.1 (C-β) and 116.2 (C-α). The proposed structure was

confirmed by the HMBC correlations of H-2 to C-4, C-6, C-β; H-β to C-2, C-α and H-α to C-1, C=O (Mounnissamy, Kavimani, Quine & Subramani, 2011).

Table 4-10 The NMR Spectral Data of Compound **12**

Position	12 *			Mounnissamy et al., 2011 **	
	δ_H (*mult.*, J_{Hz})	δ_C	HMBC	δ_H (*mult.*, J_{Hz})	δ_C
1	-	128.1 (C)		-	126.19
2	7.15 (1H, *d*, 2.1)	115.7 (CH)	C-4, C-6, C-β	6.99 (1H, *d*, 2.3)	115.59
3	-	147.2 (C)	-	-	146.05
4	-	149.3 (C)	-	-	145.16
5	6.86 (1H, *d*, 8.1)	116.9 (CH)	C-1, C-3	6.72 (1H, *d*, 8.4)	116.25
6	7.03 (1H, *d*, 8.1, 2.1)	122.9 (CH)	-	6.92 (1H, *dd*, 8.4, 2.3)	121.79
α	6.28 (1H, *d*, 15.9)	116.2 (CH)	C-1, C=O	7.38 (1H, *d*, 16.0)	115.00
β	7.53 (1H, *d*, 15.9)	146.1 (CH)	C-2, C-α	6.15 (1H, *d*, 16.0)	148.64
C=O	-	168.0 (C)	-	-	168.51

Note. *300 MHz in acetone-d_6
**500 MHz in DMSO-d_6

Compound **13** (5,7,4′-trihydroxyflavone, apigenin) was isolated as a yellow solid, m.p. 344-348 °C. The UV spectrum in MeOH exhibited maximum absorption bands (log ε) at 206.6 (4.34), 267.9 (4.08) and 333.0 (4.11) nm. The IR (KBr) spectrum showed the absorption bands of stretching of hydroxyl (3429cm^{-1}) and carbonyl (1631 cm^{-1}) groups. The ^{1}H NMR spectrum (table 4-11) indicated a flavone characteristic by the appearance of a *singlet* signal of a methine proton (H-3) at δ 6.50. The signal of a chelated hydroxyl group 5-OH (12.88, *s*) and the signals of AA′BB′ system of H-2′, H-6′ (δ 7.81, *d*, J = 8.7 Hz) and H-3′, H-5′ (δ 6.90, *d*, J = 8.7 Hz) were displayed in the spectrum. In addition, the resonances of *meta* coupling of H-6 and H-8 were detected at δ 6.12 (*d*, J = 2.1 Hz) and 6.41 (*d*, J = 2.1 Hz), respectively. The proof of vinylic proton H-3 was obtained from the results of ^3J cross peaks of H-3 to C-4a (δ 105.5) and C-1′ (δ 123.3) and ^2J cross peak of H-3 to C-2 (δ 165.1) and C-4 (δ 183.1) (Jeong, G.-S., Lee, Jeong, S.-N., Kim, Y.-C.& Kim, E.-C., 2009).

CHAPTER 5
CONCLUSION

In conclusion, the phytochemical investigation of the flowers, leaves, roots, stems and twigs of *Cassia alata* Linn. had led to the isolation and identification of twenty three known compounds. Six compounds [hydroxyquinol (**1**), 2′,6′-dihydroxy-4′-methoxydihydrochalcone (**2**), stigmasterol (**3**), ziganein (**4**), aloe emodin (**5**), and emodin (**6**)] were isolated from the flowers and two compounds [kaempferol (**7**) and diosmetin (**8**)] were obtained from the leaves. Eight compounds [physcion (**9**), *β*-sitosterol (**10**), lupeol (**11**), caffeic acid (**12**), apigenin (**13**), *trans*-resveratrol (**14**), ω-hydroxyemodin (**15**), and orientalone (**16**)] were isolated from the roots, two compounds [euxanthone (**17**) and 3-geranyloxy-1,7-dihydroxyxanthone (**18**)] were obtained from the stems and five compounds [*trans*-dihydrokaempferol (**19**), luteolin (**20**), lunatin (**21**), 7,4′-dihydroxy-5-methoxyflavone (**22**), and hydroquinone (**23**)] were isolated from the twigs. Moreover, Sixteen compounds (**1, 2, 4, 8, 9, 11-19, 21,** and **22**) were reported for the first time instance as constituents of *C. alata* Linn.

The crude extracts of *C. alata* showed moderate antibacterial activity against gram-positive (*Bacillus cereus* TISTR 687, *Staphylococcus aureus* TISTR 1466 and methicillin resistant *Staphylococcus aureus* (MRSA)-SK1) and weak antibacterial activity against gram-negative (*Escherichia coli* TISTR 780, *Pseudomonas aeruginosa* TISTR 781 and *Salmonella typhimurium* TISTR 292). Compounds **2** and **6** exhibited strong antibacterial activity against *Bacillus cereus* TISTR 687 and methicillin resistant *Staphylococcus aureus* SK1with MICs values of 8 and 4 μg/mL, respectively. Whereas, the dichloromethane and acetone extracts of *C. alata* stems showed inactive anticancer against KB-oral carvity cancer, NCI-H187 small cell lung cancer, and MCF7-Breast cancer. In addition, kaempferol (**7**) showed antioxidative activity (IC_{50} 9.67 ± 0.29 μM) that was three times stronger than that of ascorbic acid (IC_{50} 25.41 ± 0.92 μM). *trans*-Resveratrol (**14**) showed moderate antioxidative activity (IC_{50} 45.90 ± 0.22 μM), which was almost better than BHT (IC_{50} 46.56 ± 0.45 μM).

Table 4-11 The NMR Spectral Data of Compound **13**

Position	13 *			Jeong et al., 2009 **	
	δ_H (*mult.*, J_{Hz})	δ_C (DEPT)	HMBC	δ_H (*mult.*, J_{Hz})	δ_C
2	-	165.1 (C)	-	-	163.7
3	6.50 (1H, *s*)	104.1 (CH)	C-2, C-4, C-4a, C-1′	6.78 (1H, *s*)	102.8
4	-	183.1 (C)	-	-	181.4
4a	-	105.5 (C)	-	-	103.7
5	-	163.4(C)	-	-	161.2
6	6.12 (1H, *d*, 2.1)	99.7 (CH)	C-4a, C-5, C-7, C-8	6.36 (1H, *d*, 2.0)	98.9
7	-	165.0 (C)	-	-	164.3
8	6.41 (1H, *d*, 2.1)	94.7 (CH)	C-4a,C-6, C-7, C-8a	6.49 (1H, *d*, 2.0)	94.0
8a	-	158.8 (C)	-	-	157.3
1′	-	123.3 (C)	-	-	121.2
2′,6′	7.81 (2H, *d*, 8.7)	129.2 (CH)	C-2, C-2′,6′, C-4′	7.93 (2H, *d*, 8.8)	128.4
3′,5′	6.90 (2H, *d*, 8.7)	116.9 (CH)	C-1′, C-3′,5′, C-4′	6.94 (2H, *d*, 8.8)	116.0
4′	-	161.9 (C)	-	-	161.4
5-OH	12.88 (1H, *s*)	-	C-4a, C-5, C-6	12.98 (1H, *s*)	-
7-OH	-	-	-	10.59 (1H, *s*)	-

Note. *300 MHz in acetone-d_6
**400 MHz in DMSO-d_6

Compound **14** (*trans*-3,5,4′-trihydroxystilbene, *trans*-resveratrol) was obtained as a pale yellow solid, m.p. 260.9-261.8 °C and its (HR)-EI-MS gave an $[M]^+$ ion peak at *m/z* 228.078 (calcd for $C_{14}H_{12}O_3$). The UV spectrum in MeOH exhibited maximum absorptions (log ε) at 216.7 (4.49), 305.6 (4.54) and 321.5 (4.53) nm. The IR (KBr) spectrum showed the stretching ofhydroxyl (3234 cm^{-1}) and alkene (1605 cm^{-1}) groups. The ^1H NMR spectrum (table 4-12) showed signals due to four aromatic protons for an AA′BB′ pattern at δ 7.41 (2H, *d*, J = 8.7 Hz, H-2′,6′), 6.84 (2H, *d*, J = 8.7 Hz, H-3′,5′) and AMX pattern of H-2,6 at δ6.54 (2H, *d*, J = 2.1 Hz) and H-4 at δ6.27 (1H, *t*, J = 2.1 Hz). The coupling constant (J = 16.2 Hz) of H-α (δ6.88, 1H, *d*) and H-α′ (δ7.02, 1H, *d*) implied that the geometry of this compound was *trans*. The ^{13}C NMR spectrum showed three oxygenated aromatic carbons at δ 159.6 (×2, C-3,5) and 158.2 (C-4′); two quaternary carbons at δ141.0 (C-1) and 130.1 (C-1′); seven

methine carbons at δ 105.7 (×2, C-2,6), 102.7 (C-4), 128.8 (×2, C-2′,6′) and 116.5 (×2, C-3′,5′); and two olefinic carbons at δ 126.9 (C-α) and 129.2 (C-α'). The positions of *meta* aromatic protons H-2,6 and H-4 were confirmed by the HMBC correlations of H-2,6 to C-2,6, C-3,5, C-4, C-α and H-4 to C-2,6, C-3,5. The correlations of H-α to C-1, C-2,6, C-3,5, C-1′, C-$\alpha'$and H-α' to C-1, C-1′, C-2′,6′, C-α supported the stilbene pattern. The HMBC correlations of H-2′,6′ to C-2′,6′, C-3′,5′, C-4′, C-α' and H-3′,5′ to C-1′, C-2′,6′, C-3′,5′, C-4′, C-α' confirmed the positions of AA′BB′pattern (Lee et al., 2009).

Table 4-12 The NMR Spectral Data of Compound **14**

Position	14 *			Lee et al., 2009 **	
	δ_H (*mult.*, J_{Hz})	δ_C (DEPT)	HMBC	δ_H (*mult.*, J_{Hz})	δ_C
1	-	141.0 (C)	-	-	141.3
2,6	6.54 (2H, *d*, 2.1)	105.7 (CH)	C-2,6, C-3,5, C-4, C-α	6.44 (2H, *br s*)	105.8
3,5	-	159.6 (C)	-	-	159.7
4	6.27 (1H, *t*, 2.1)	102.7 (CH)	C-2,6, C-3,5	6.16 (1H, *br s*)	102.6
1′	-	130.1 (C)	-	-	130.4
α	6.88 (1H, *d*, 16.2)	126.9 (CH)	C-1, C-2,6, C-3,5, C-1′, C-α'	6.79 (1H, *d*, 16.0)	127.0
α'	7.02 (1H, *d*, 16.2)	129.2 (CH)	C-1, C-1′, C-2′,6′, C-α	6.95 (1H, *d*, 16.0)	129.4
2′,6′	7.41 (2H, *d*, 8.7)	128.8 (CH)	C-2′,6′, C-3′,5′, C-4′, C-α'	7.35 (2H, *d*, 8.0)	128.8
3′,5′	6.84 (2H, *d*, 8.7)	116.5 (CH)	C-1′, C-2′,6′, C-3′,5′, C-4′,C-α'	6.76 (2H, *d*, 8.0)	116.5
4′	-	158.2 (C)	-	-	158.4

Note. *300 MHz in acetone-d_6

**400 MHz in CD_3OD

Compound **15** (1,6,8-trihydroxy-3-(hydroxymethyl)anthraquinone or ω-hydroxyemodin or citreorosein) was obtained as an orange solid, m.p. 280-285 °C, (287-289 °C, Fujimoto, Nakamura, Okuyama & Ishibashi, 2004). The UV spectrum (in MeOH) exhibited maximum absorptions (log ε) at 221.5 (4.43), 250.7 (4.16), 265.7 (4.15), 288.7 (4.19) and 435.8 (3.96) nm. The IR (KBr) spectrum showed the stretching of hydroxyl (3419 cm^{-1}) and carbonyl (1628 cm^{-1}) groups. The ^1H NMR spectral data (table 4-13) showed signals of two chelated hydroxyl protons at δ12.14 (1-OH) and 12.20 (8-OH) and a hydroxymethyl proton at δ4.79 (3-C$\underline{H_2}$OH). The spectrum further showed the resonances of two sets of *meta* coupled protons H-2, H-4 and H-5, H-7 at δ7.33 (*br s*), 7.77 (*br s*) and 7.28 (*d*, J = 2.4 Hz), 6.68 (*d*, J = 2.4 Hz), respectively. The HMBC correlations of 3-C$\underline{H_2}$OH to C-2, C-3 and C-4 indicated the position of hydroxymethyl group at C-3. The positions of two chelated hydroxyl groups were confirmed by the correlations of 1-OH to C-1, C-2, C-9a and 8-OH to C-7, C-8, C-8a (Fujimoto et al., 2004).

Table 4-13 The NMR Spectral Data of Compound **15**

Position	15 *			Fujimoto et al., 2004	
	δ_H (*mult.*, J_{Hz})	δ_C (DEPT)	HMBC	δ_H (*mult.*, J_{Hz})	δ_C
1	-	164.0 (C)	-	-	162.6 (*s*)
2	7.33 (1H, *br s*)	121.8 (CH)	3-C$\underline{H_2}$OH, C-4, C-9a	7.32 (*br s*)	120.9 (*d*)
3	-	154.0 (C)	-	-	153.1 (*s*)
4	7.77 (1H, *br s*)	118.2 (CH)	C-2, 3-C$\underline{H_2}$OH, C-9a, C-10	7.76 (*br s*)	117.3 (*d*)
4a	-	134.0 (C)	-	-	131.2 (*s*)
5	7.28 (1H, *d*, 2.4)	110.1 (CH)	C-7, C-10	7.28 (*d*, 2.4)	109.0 (*d*)
6	-	167.0 (C)	-	-	165.5 (*s*)
7	6.68 (1H, *d*, 2.4)	109.0 (CH)	C-5, C-8a	6.68 (*d*, 2.4)	108.1 (*d*)
8	-	167.0 (C)	-	-	165.7 (*s*)
8a	-	111.0 (C)	-	-	109.7 (*s*)
9	-	191.8 (C)	-	-	191.0 (*s*)
9a	-	114.0 (C)	-	-	114.4 (*s*)
10	-	182.5 (C)	-	-	181.4 (*s*)
10a	-	137.0 (C)	-	-	133.6 (*s*)
1-OH	12.14 (1H, *s*)	-	C-1, C-2, C-9a	12.14 (*s*)	-

6-OH	-	-	-		-
8-OH	12.20 (1H, *s*)	-	C-7, C-8, C-8a	12.20 (*s*)	-
3-CH_2OH	4.79 (2H, *s*)	63.8 (CH_2)	C-2, C-3, C-4	4.79 (2H, *s*)	62.9 (*t*)

Note. *300 MHz in acetone-d_6
**400 MHz in CD_3OD

Compound **16** (2-methoxystypandrone, orientalone) was isolated as a yellow solid. The strong absorption bands (log ε) at 224 (4.51), 288 (4.13), and 422 (3.69) nm were detected on UV spectrum in MeOH. The IR spectrum (KBr) showed maximum absorption bands at 3411 cm^{-1} (O-H stretching) and 1713 cm^{-1} (C=O stretching). The ^1H NMR spectral data (table 4-14) exhibited a *singlet* signal of an olefinic proton H-3 at δ 6.29, a *broad singlet* signal of a chelated hydroxy 5-OH at δ 13.01, a *singlet* signal of an aromatic proton H-8 at δ 7.46. The remaining signals are a *singlet* signal of a methoxy 2-OCH_3 at δ 3.97, a *singlet* signal of an acetyl 6-$CO\underline{C}H_3$ at δ 2.53, and a *singlet* signal of a methyl 7-CH_3 at δ 2.34. These assignment were confirmed by HMBC correlations of 2-OCH_3 to C-2; H-3 to C-1, C-2, C-4, C-4a; 6-$COC\underline{H}_3$ to C-6, 6-$\underline{C}OCH_3$; 7-CH_3 to C-6, C-7, C-8, C-8a and H-8 to C-1, C-4a, C-6, 7-$\underline{C}H_3$, respectively (Nishina, Kubota & Osawa, 1993).

Table 4-14 The NMR Spectral Data of Compound **16**

Position	**^1H NMR** (400 MHz, Acetone-d_6)	**^{13}C NMR** (100 MHz, Acetone-d_6)	**HMBC**
1	-	179.4 (C)	-
2	-	162.5 (C)	-
3	6.29 (1H, *s*)	110.2 (CH)	C-1, C-2, C-4, C-4a
4	-	191.9 (C)	-
4a	-	113.0 (C)	-
5	-	159.3 (C)	-
6	-	139.0 (C)	-
7	-	143.8 (C)	-
8	7.46 (2H, *s*)	121.6 (CH)	C-1, C-4a, C-6, 7-CH_3
8a	-	135.0 (C)	-
2-OCH_3	3.97 (3H, *s*)	57.3 (CH_3)	C-2

5-OH	13.02 (1H, *br s*)	-	-
6-$\underline{C}OCH_3$	-	202.9 (C)	-
6-$CO\underline{C}H_3$	2.53 (3H, *s*)	31.8 (CH_3)	C-6
7-CH_3	2.34 (3H, *s*)	19.7 (CH_3)	C-6, C-7, C-8, C-8a

Compound **17** (1,7-dihydroxyxanthone, euxanthone) was obtained as a yellow solid, m.p. 239-241°C, (241-242°C, Kang & Xu, 2008). The UV spectrum (in MeOH) exhibited maximum absorptions (log ε) at 203.5 (3.72) and 225.0 (3.27) nm. The IR (KBr) spectrum showed the stretching of hydroxyl (3309 cm^{-1}) and carbonyl (1642 cm^{-1}) groups. The ^1H NMR spectrum (table 4-15) showed a *singlet* signal of a hydrogen-bonded hydroxyl proton 1-OH at δ 12.70. The signals of aromatic protons for an ABM pattern [H-2 (δ 6.75, *dd*, J = 8.5, 1.0 Hz), H-3 (δ 7.68, *dd*, J = 8.5 Hz) and H-4 (δ 6.98, *dd*, J = 8.5, 1.0 Hz] and an ABX pattern [H-5 (δ 7.50,*d*, J = 9.0 Hz), H-6 (δ7.41,*d*, J = 9.0, 3.0 Hz) and H-8 (δ 7.58, *d*, J = 3.0 Hz)] were displayed in the spectrum. The HMBC correlations of H-2 to C-1, C-4; H-3 to C-1, C-4a; H-4 to C-2, C-4a, C-9;H-5 toC-7, C-8a, C-10a; H-6 to C-7, C-8, C-10a and H-8 to C-6, C-9, C-10 a supported the proposed structure (Kang & Xu, 2008).

Table 4-15 The NMR Spectral Data of Compound **17**

Position	**17** *			**Kang & Xu, 2008** **	
	δ_H (*mult.*, J_{Hz})	δ_C	**HMBC**	δ_H (*mult.*, J_{Hz})	δ_C
1	-	162.8	-	-	161.0
2	6.75 (1H, *dd*, 8.5,1.0)	109.2	C-1, C-4	6.78 (1H, *dd*, 8.1, 0.8)	109.7
3	7.68 (1H, *t*, 8.5)	137.9	C-1, C-4a	7.71 (1H,*t*, 8.3)	137.3
4	6.98 (1H, *dd*,8.5, 1.0)	107.9	C-2, C-4a, C-9	7.02 (1H, *dd*, 8.3,0.8)	108.0
4a	-	157.4	-	-	154.2
5	7.50 (1H, *d*, 9.0)	120.3	C-7, C-8a, C-10a	7.54 (1H, *d*, 9.1)	119.5
6	7.41 (1H,*dd*, 9.0, 3.0)	126.3	C-7, C-8, C-10a	7.44 (1H,*dd*, 9.0,3.0)	125.6
7	-	155.2	-	-	155.9
8	7.58 (1H, *d*, 3.0)	109.2	C-6, C-9, C-10a	7.62 (1H, *d*, 3.0)	107.9
8a	-	121.9	-	-	120.5
9	-	183.1	-	-	181.7

9a	-	110.6	-	-	107.2
10a	-	151.1	-	-	149.4
1-OH	12.70 (1H, *s*)	-	C-1, C-2, C-9a	12.73 (1H, s)	-

Note. *400 MHz in acetone-d_6
**400 MHz in DMSO-d_6

Compound **18** (3-geranyloxy-1,7-dihydroxyxanthone) was obtained as a yellow solid and its EI-MS gave an $[M]^+$ ion peak at *m/z* 380.7 corresponding to the molecular formula $C_{23}H_{24}O_5$. The UV spectrum in MeOH exhibited maximum absorptions (log ε) at 236.9 (3.60), 259.9 (3.74), 308.2 (3.35), and 374.1 (2.96) nm. The IR (KBr) spectrum showed the stretching of hydroxyl (3308 cm^{-1}) and carbonyl (1661 cm^{-1}) groups. The ^1H NMR spectrum (table 4-16) showed signals of a chelated hydroxyl proton at δ 12.92 (1-OH) and a free hydroxyl proton at δ 8.96 (7-OH). The signals of ABX aromatic protons [H-5 (δ 7.46, *d*, *J* = 9.0 Hz), H-6 (δ 7.37, *dd*, *J* = 2.7, 9.0 Hz), H-8 (δ 7.57, *d*, *J* = 2.7 Hz)] and *meta* coupled protons [H-2 (δ 6.32, *d*, *J* = 2.1 Hz) and H-4 (δ 6.52, *d*, *J* = 2.1 Hz)] were displayed in the spectrum. Moreover, the presence of an oxygeranyl unit was observed from the characteristic signals of geranyl side chain at δ 4.75 (2H, *d*, *J* = 6.6 Hz, H-1′), 5.50 (1H, *d*, *J* = 2.7 Hz, H-2′), 2.25 (2H, *m*, H-4′), 2.25 (2H, *m*, H-5′), 5.12 (1H, *t*-like, H-6′), 1.60 (3H, *br s*, H-8′), 1.80 (3H, *br s*, H-9′) and 1.65 (3H, *br s*, H-10′). The evidences from the chemical shift of H-1′ (δ 4.75) and C-3 (δ 167.6) indicated that the geranyl side chain attached to an oxygen atom (Boonnaket al., 2009).

Table 4-16 The NMR Spectral Data of Compound **18**

Position	18 *			Boonnak et al., 2009 **	
	δ_H (*mult.*, J_{Hz})	δ_C (DEPT)	HMBC	δ_H (*mult.*, J_{Hz})	δ_C
1	-	164.8 (C)	-	-	163.2
2	6.32 (1H, *d*, 2.1)	98.8 (CH)	C-1, C-3, C-4, C-9a	6.34 (1H, *d*, 2.4)	97.6
3	-	167.6 (C)	-	-	166.1
4	6.52 (1H, *d*, 2.1)	94.3 (CH)	C-2, C-3, C-4a, C-9a	6.40 (1H, *d*, 2.4)	93.2
4a	-	159.3 (C)	-	-	157.8
5	7.46 (1H, *d*, 9.0)	120.5 (CH)	C-6, C-9a, C-10a	7.30 (1H,*d*, 9.3)	118.9

6	7.37 (1H,*dd*,9.0, 2.7)	125.8 (CH)	-	7.26 (1H, *d*, 9.3)	124.2
7	-	155.5 (C)	-	-	152.5
8	7.57 (1H, *d*, 2.7)	109.9 (C)	C-6, C-10a	7.59 (1H, *br s*)	109.0
8a	-	122.4 (C)	-	-	120.9
9	-	181.9 (C)	-	-	180.5
9a	-	104.6 (C)	-	-	103.5
10a	-	151.4 (C)	-	-	150.5
1′	4.75 (2H, *d*, 6.6)	67.0 (CH_2)	C-3, C-2′	4.63 (1H, *d*, 6.6)	65.6
2′	5.50 (1H, *br t*, 6.6)	120.3 (CH)	C-4′, C-9′	5.50 (1H, *brt*, 6.6)	118.3
3′	-	142.9 (C)	-	-	142.3
4′	2.25 (2H, *m*)	40.7 (CH_2)	C-5′, C-9′	2.13 (2H, *m*)	39.5
5′	2.25 (2H, *m*)	27.5 (CH_2)	C-6′	2.10 (2H, *m*)	26.2
6′	5.12 (1H, *t*-like)	125.2 (CH)	-	5.11 (1H, *brt*, 5.7)	123.6
7′	-	132.7 (C)	-	-	131.9
8′	1.65 (3H, *br s*)	26.3 (CH_3)	C-6′, C-7′, C-10′	1.69 (3H, *s*)	25.6
9′	1.80 (3H, *br s*)	17.3 (CH_3)	C-2′, C-3′, C-4′	1.78 (3H, *s*)	16.7
10′	1.60 (3H, *br s*)	18.3 (CH_3)	C-6′, C-7′, C-8′	1.62 (1H, *s*)	17.7
1-OH	12. 92 (1H, *s*)	-	-	12.72 (1H, *s*)	-
7-OH	8.96 (1H *br s*)	-	-	7.03 (1H, *brs*)	-

Note. *300 MHz in acetone-d_6

**300 MHz in $CDCl_3$

Compound **19** (5,7,4′-trihydroxydihydroflavonol, *trans*-dihydrokaempferol) was obtained as a yellow solid, this compound exhibited UV maximum absorption bands in MeOH (log ε) at 215.1 (4.34), and 291.4 (4.05) nm, a characteristic of a flavone nucleus. The IR (KBr) spectrum showed the absorption bands at 3278 cm^{-1} (a hydroxyl group) and 1640 cm^{-1} (a carbonyl group). The ^1H NMR spectrum (table 4-17) showed characteristic resonances of a *trans*-dihydroflavonol protons at δ 5.08 (1H, *d*, J =11.7 Hz, H-2) and 4.65 (1H, *d*, J =11.7 Hz, H-3). The presence of a hydrogen-bonded hydroxyl proton was at δ 11.71 (1H, *s*, 5-OH). The spectrum further showed the resonances of *meta* coupled protons at δ 5.99 (1H, *d*, J = 1.8 Hz, H-6) and 5.95 (1H, *d*, J = 1.8 Hz, H-8) and a AA′BB′ pattern at δ 7.42 (2H, *d*, J = 8.1 Hz, H-2′,6′), δ 6.89 (2H, *d*, J = 8.1 Hz, H-3′,5′). The H-2 signal was indicated from the

results of ^{2}J and ^{3}J cross peaks of H-2 to C-3 (δ 73.6), C-4 (δ 198.7), C-1′ (δ 129.6) and C-2′,6′ (δ 130.8) in the HMBC experiment (Xiang, Su, Hu & Yan, 2011).

Table 4-17 The NMR Spectral Data of Compound **19**

Position	19 *			Xiang et al., 2011 **	
	δ_H (*mult.*, J_{Hz})	δ_C (DEPT)	HMBC	δ_H (*mult.*, J_{Hz})	δ_C
2	5.08 (1H, *d*, 11.7)	84.9 (CH)	C-3, C-4, C-1′, C-2′,6′	5.09 (1H, *d*, 11.6)	84.4
3	4.65 (1H,*d*, 11.7)	73.6 (CH)	C-2	4.66 (1H, *d*, 11.6)	73.1
4	-	198.7 (C)	-	-	198.2
4a	-	102.0 (C)	-	-	101.5
5	-	165.5 (C)	-	-	165.0
6	5.99 (1H,*d*, 1.8)	97.6 (CH)	C-4a, C-5, C-7, C-8	5.96 (1H, *d*, 2.0)	97.2
7	-	168.5 (C)	-	-	167.9
8	5.95 (1H, *d*, 1.8)	96.6 (CH)	C-4a, C-6, C-7, C-8a	6.00 (1H, *d*, 2.0)	96.1
8a	-	164.7 (C)	-	-	164.2
1′	-	129.6 (C)	-	-	129.1
2′,6′	7.42 (2H, *d*, 8.1)	130.8 (CH)	C-2, C-1′, C-2′,6′	7.42 (2H, *d*, 8.6)	130.2
3′,5′	6.89 (2H, *d*, 8.1)	116.4 (CH)	C-1′, C-3′,5′, C-4′	6.90(2H, *d*, 8.6)	115.9
4′	-	159.4 (C)	-	-	158.8
5-OH	11.71 (1H, *s*)	-	C-4a, C-5, C-6	-	-

Note. *300 MHz in acetone-d_6
**400 MHz in acetone-d_6

Compound **20** (5,7,3′,4′-tetrahydroxyflavone or luteolin) was obtained as a yellow needle, m.p. 325-327 °C (327-329 °C, Miyazawa & Hisama, 2003). The UV spectrum (in MeOH) exhibited maximum absorptions (log ε) at 207.5 (4.65), 253.9 (4.34), 268.0 (4.29) and 348.4 (4.41) nm. The IR (KBr) spectrum showed the stretching of hydroxyl (3401 cm^{-1}) and carbonyl (1662 cm^{-1}) functional groups. The ^{1}H NMR spectrum (table 4-18) revealed a *singlet* of a flavone type vinylic proton (H-3) at δ 6.45, a *singlet* of a chelated hydroxyl group (4-OH) at δ 12.85 and three *broad singlet* signals of three free hydroxy groups at δ 10.05 (7-OH), 8.98 (3′-OH) and 8.69 (4′-OH). The signals of ABM system of H-2′ (δ 7.36, *d*, J = 2.1 Hz), H-5′ (δ 6.86, *d*, J = 8.4 Hz) and H-6′ (δ 7.32, *d*, J = 2.1, 8.4 Hz) were displayed in the spectrum. Two

doublets at δ 6.11 and 6.39 with a coupling constant of 2.1 Hz represented the H-6 and H-8, respectively. The proof of a vinylic proton H-3 was obtained from the results of ^3J cross peaks of H-3 to C-4a (δ 104.3) and C-1′ (δ 122.7) and ^2J cross peaks of H-3 to C-2 (δ 164.4) and C-4 (δ182.2) (Miyazawa & Hisama, 2003).

Table 4-18 The NMR Spectral Data of Compound **20**

Position	10 (300 MHz in acetone-d_6)			Miyazawa & Hisama, 2003	
	δ_H (*mult.*, J_{Hz})	δ_C	HMBC	δ_H (*mult.*, J_{Hz})	δ_C
2	-	164.4	-	-	164.3
3	6.45 (1H, *s*)	103.2	C-2, C-4, C-4a, C-1′	6.68 (1H, *s*)	-
4	-	182.2	-	-	181.6
4a	-	104.3	-	-	105.2
5	-	162.4	-	-	161.0
6	6.11 (1H, *d*, 2.1)	98.9	C-4a, C-5, C-7, C-8	6.40 (1H, *d*, 2.0)	100.0
7	-	164.3	-	-	162.8
8	6.39 (1H, *d*, 2.1)	93.9	C-4, C-4a, C-6, C-7, C-8a	6.76 (1H, *d*, 2.0)	94.7
8a	-	157.9	-	-	156.8
1′	-	122.7	-	-	121.3
2′	7.36 (1H, *d*, 2.1)	115.7	C-2, C-1′, C-3′, C-6′	7.39 (1H, *d*, 2.2)	113.5
3′	-	145.8	-	-	145.6
4′	-	149.4	-	-	149.7
5′	6.86 (1H, *d*, 8.4)	113.2	C-1′, C-3′, C-4′	6.89 (1H, *d*, 9.0)	115.9
6′	7.32 (1H, *d*, 8.4, 2.1)	119.1	C-2, C-4′, C-5′	7.41 (1H, *dd*, 9.0, 2.2)	118.9
5-OH	12.85 (1H, *s*)	-	C-4, C-4a,C-5, C-6, C-7	12.90 (1H, *s*)	-
7-OH	10.05 (1H, *br s*)	-	C-6, C-7, C-8	-	-
3′-OH	8.98 (1H, *br s*)	-	C-2′, C-3′, C-4′	-	-
4′-OH	8.69 (1H, *br s*)	-	C-3′, C-4′, C-5′	-	-

Compound **21** (1,3,8-trihydroxy-6-methoxyanthraquinone, lunatin) was obtained as an orange solid. The ^{1}H NMR spectral data (table 4-19) showed two *singlet*signals of two chelated hydroxyl protons at δ 12.09 (1-OH) and 12.04 (8-OH). The resonances of two sets of *meta* protons H-2 (δ 6.54, *d*, J = 2.7 Hz), H-4 (δ 7.14, *d*, J = 2.7 Hz) and H-5 (δ 7.68, *d*, J = 2.1 Hz, H-7 (δ 7.28, *d*, J = 2.1 Hz) were observed. The remaining *singlet* signal at δ 4.65 was assigned to be methoxy group at C-6 according to the ^{13}C NMR signal of methoxy carbon at δ 62.0. The HMBC correlation of 6-OCH_3 to C-6 confirmed the position of methoxy group at C-6. The position of *meta* aromatic protons H-2, H-4, H-5 and H-7 were confirmed by the correlations of H-2 to C-1, C-4; H-4 to C-2, C-10; H-5 to C-7, C-8a, C-10, 6-O$\underline{C}H_3$ and H-7 to C-5, C-8a, 6-O$\underline{C}H_3$, respectively (Jadulco et al., 2002).

Table 4-19 The NMR Spectral Data of Compound **21**

Position	21 *			Jadulco et al., 2002 **	
	δ_H (*mult.*, J_{Hz})	δ_C	HMBC	δ_H (*mult.*, J_{Hz})	δ_C
1	-	166.2	-	-	164.4
2	6.54 (1H, *d*, 2.7)	108.4	C-1, C-4	6.56 (1H, *d*,2.3)	108.1
3	-	166.8	-	-	165.9
4	7.14 (1H, *d*, 2.7)	109.3	C-2, C-10	7.09 (1H, *d*,2.3)	109.3
4a	-	134.7	-	-	134.6
5	7.68 (1H, *d*, 2.1)	118.2	C-7, C-8a, C-10, 6-OCH_3	7.14 (1H, *d*, 2.5)	107.4
6	-	153.2	-	-	165.5
7	7.19 (1H, *d*, 2.1)	120.8	C-5, C-8a, 6-OCH_3	6.86 (1H, *d*, 2.5)	106.6
8	-	163.7	-	-	164.1
8a	-	116.7	-	-	109.6
9	-	188.6	-	-	188.4
9a	-	114.6	-	-	108.3
10	-	181.1	-	-	181.1
10a	-	135.2	-	-	134.8
1-OH	12.09 (1H, *s*)	-	C-1, C-2, C-9a	12.22 (1H, *s*)	-
8-OH	12.04 (1H, *s*)	-	C-7, C-8, C-8a	12.31 (1H, *s*)	-
6-OCH_3	4.65 (3H, *s*)	62.0	C-6, C-7	3.91 (3H, *s*)	56.2

Note. *400 MHz in acetone-d_6
**600 MHz in DMSO-d_6

Compound **22** (7,4′-dihydroxy-5-methoxyflavone) was obtained as a yellow solid, m.p. 280-282 °C, (279-283 °C, Mbouangouere et al., 2007). This compound exhibited UV maximum absorption bands (in MeOH) and log ε at 206.4 (4.83), 264.0 (4.59) and 331.9 (4.67) nm, a characteristic of a flavone nucleus. The IR (KBr) spectrum showed the absorption bands at 3242 cm^{-1} (a hydroxyl group) and 1648 cm^{-1} (a carbonyl group). The ^{1}H NMR spectrum (table 4-20) showed the characteristic resonances of a flavone proton at δ 6.50 (1H, *s*, H-3). The presence of a methoxy group was shown in the spectrum, of which the *singlet* signal at δ 3.93 (3H, *s*, 5-OCH_3). The spectrum further exhibited the resonances of *meta* coupled protons at δ 6.55 (1H, *d*, J = 1.8 Hz, H-6) and 6.37 (1H, *d*, J = 1.8 Hz, H-8) and a AA′BB′ pattern at δ 7.72 (2H, *d*, J = 8.7 Hz, H-2′,6′), δ 6.95 (2H, *d*, J = 8.7 Hz, H-3′,5′). The proof of a vinylic proton H-3 was obtained from the results of $^{2}J$and ^{3}J cross peaks of H-3 to C-2 (δ 164.8), C-4 (δ 182.0) and C-1′ (δ 125.0) in the HMBC experiment. In addition, the correlation of 5-OCH_3 to C-5 indicated the position of the methoxy group at C-5 (Mbouangouere et al., 2007).

Table 4-20 The NMR Spectral Data of Compound **22**

Position	22 *			Mbouangouere et al., 2007 **
	δ_H (*mult.*, J_{Hz})	δ_C	HMBC	δ_H (*mult.*, J_{Hz})
2	-	163.0	-	-
3	6.50 (1H, *s*)	112.0	C-2, C-4, C-4a, C-1′	6.34 (*s*)
4	-	182.0	-	-
4a	-	111.0	-	-
5		165.1	-	-
6	6.55 (1H, *d*, 1.8)	100.6	C-4a, C-5, C-7, C-8	7.61 (*d*, 2.8)
7	-	166.0	-	-
8	6.37 (1H, *d*, 1.8)	101.2	C-4a, C-6, C-7, C-8a	6.76 (*d*, 2.8)
8a	-	165.7	-	-
1′	-	128.0	-	-
2′,6′	7.72 (2H, *d*, 8.7)	132.4	C-2′,6′, C-4′	6.26 (*dd*, 8.8, 2.1)
3′,5′	6.95 (2H, *d*, 8.7)	120.8	C-1′, C-3′,5′, C-4′	6.09 (*dd*, 8.8, 2.1)
4′	-	165.0	-	-
7-OH	10.04 (1H, *s*)	-	C-6, C-7, C-8	

4′-OH	9.54 (1H, *s*)	-	C-3′,5′, C-4′	-
5-OCH_3	3.93 (3H, *s*)	61.0	C-5	3.70 (*s*)

Note. *300 MHz in $CDCl_3$+DMSO-d_6
**400 MHz in $CDCl_3$

Compound **23** (1,4-dihydroxybenzene, hydroquinone) was obtained as a yellow solid. The UV spectrum in MeOH exhibited maximum absorption bands (log ε) at 204 (4.79) and 254 (4.70) nm. The IR spectrum (KBr) showed absorption bands at 3387 cm^{-1} (O-H stretching). The ^{1}H NMR spectral data (table 4-21) exhibited two *doublet* signals at δ 7.91 (2H, *d*, J = 8.4 Hz, H-2,6) and δ 6.91 (2H, *d*, J = 8.4 Hz, H-3,5). The resulting structure was corresponded with ^{13}C NMR spectral data and confirmed by HMBC correlations.

Table 4-21 The NMR spectral data of Compound **23**

Position	**^{1}H NMR**(400 MHz, Acetone-d_6)	**^{13}C NMR**(100 MHz, Acetone-d_6)	**HMBC**
1	-	168.0	-
2,6	7.91 (2H, *d*, J = 8.4 Hz)	122.6	C-1, C-3,5, C-4
3,5	6.91 (2H, *d*, J = 8.4 Hz)	132.6	C-2,6, C-4
4	-	162.2	-

4.2 Evaluation of Antibacterial Activity

4.2.1 Antibacterial Activity of Crude Extracts

The crude extracts of *C. alata* (FD, FA, FM, LD, LA, RA, SD, SA, TD and TA) was tested for antibacterial activity against gram-positive (*Bacillus cereus* TISTR 687, methicillin resistant *Staphylococcus aureus* SK1, *Staphylococcus aureus* TISTR 1466) and gram-negative (*Escherichia coli* TISTR 780, *Pseudomonas aurenginosa* TISTR 781, *Salmonellae typhimurium* TISTR 292) by Broth Microdilution Method (CLSI, 2002). In screening result, the crudes FD, FA, LA, RA, SA, TD, and TA were able to inhibit the growth of gram-positive and gram-negative bacteria. Crudes FM, and LD were not able to inhibit growth of *S. aureus* and the crude SD was not able to inhibit growth of *S. typhimurium*. The crude extracts that showed the positive screening results were selected for determining the Minimum Inhibition Concentrations (MICs) values.

The MIC values of *C. alata* (no. 1-10) crude extracts determined by Broth Microdilution Method were summarized in table 4-22. Crude RA, FD and SD extracts exhibited strong antibacterial activity against *B. cereus* with MICs values of 40, 80 and 80 µg/mL, respectively. SA extract showed higher antibacterial activity against MRSA with MICs value of 80 µg/mL. Moreover, crude SA exhibited strong antibacterial activity against *S. aureus* at 80µg/mL. The crude FA, LA, SD, SA, TD, RA, and TA extracts showed moderate antibacterial activity against gram-positive in range of MICs 160-320 µg/mL. All crude extracts (no. 1-10) showed weak antibacterial activity against gram-negative bacteria with MICs value of 640-1280 µg/mL. Therefore, the crude extracts of *C. alata* (no. 1-10) exhibited stronger antibacterial activity against gram-positive than gram-negative. The reason for different sensitivity of gram-positive and gram-negative bacteria could be described to the morphological differences between these microorganisms. Gram-negative bacteria having an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes. The gram-positive give more susceptible having only an outer peptidoglycan layer that is not an effective permeability barrier (Scherrer & Gerhardt, 1971).

Table 4-22 MICs Values of *C. alata* Crude Extracts

No.	Sample	Antibacterial activity (MICs, µg/mL)					
		Gram-positive			Gram-negative		
		B.C	MRSA	S.A	E.C	Ps.A	S.T
1	Crude FD	80	160	320	640	1280	1280
2	Crude FA	160	1280	640	640	1280	1280
3	Crude FM	640	1280	-	640	1280	1280
4	Crude LD	1280	1280	-	640	1280	1280
5	Crude LA	160	320	160	640	1280	1280
6	Crude RA	40	160	160	640	1280	1280
7	Crude SD	80	320	640	640	1280	-
8	Crude SA	320	80	80	640	1280	1280
9	Crude TD	320	1280	1280	640	640	1280
10	Crude TA	160	320	320	640	1280	1280
11	Gentamicin	-	-	-	0.5	1	0.5
12	Vancomycin	0.5	0.5	0.5	-	-	-

4.2.2 Antibacterial Activity of Pure Compounds

Some of the pure compounds obtained from each extract were evaluated for their antibacterial activity against gram-positive (*Bacillus cereus* TISTR 687, methicillin resistant *Staphylococcus aureus* SK1, *Staphylococcus aureus* TISTR 1466) and gram-negative (*Escherichia coli* TISTR 780, *Pseudomonas aurenginosa* TISTR 781, *Salmonellae typhimurium* TISTR 292) by Broth Microdilution Method. All isolated compounds which were shown the positive screening results were further selected to determine the Minimum Inhibition Concentrations (MICs) values.

The MICs values of the selected compounds were summarized in table 4-23. Compounds **2** and **6** showed strong antibacterial activity against *B. cereus* and MRSA with MICs values of 8 and 4 µg/mL, respectively. Compounds **1** and **2** exhibited moderate antibacterial activity against MRSA in the range of MICs values of 16-32 µg/mL. Compound **6** exhibited antibacterial activity against *B. cereus* and *S. aureus*

with MICs value of 16 μg/mL. In addition, compounds **4**, **5**, **7**, **9**, **14**, **15**, and **20** showed weak antibacterial activity against gram-positive bacteria with MICs range of 64-128 μg/mL. This strong antibacterial activity of emodin (**6**) against MRSA was supported by antimicrobial activity of emodin isolated from *Rheum palmatum* L., and the compound showed antimicrobial activity against 17 different strains of MRSA with the minimum inhibitory concentrations (MICs) in the range of 1.5-25 μg/mL (Lee *et al.*, 2010). All isolated compounds showed weak antibacterial activity against gram-negative bacteria (MICs 64-128 μg/mL) which were corresponded to the results of antibacterial activity against gram-negative bacteria of the crude extracts (MICs 640-1280 μg/mL) as shown in table 4-22.

Table 4-23 MICs Values of Pure Compounds from *C. alata*

No.	Sample	Antibacterial activity (MICs, μg/mL)					
		Gram-positive			Gram-negative		
		B.C	MRSA	S.A	E.C	Ps.A	S.T
1	**1**	64	16	-	128	64	64
2	**2**	8	32	-	128	64	128
3	**4**	128	-	-	-	64	128
4	**5**	128	128	128	128	128	128
5	**6**	16	4	16	-	128	128
6	**7**	128	128	128	128	128	128
7	**9**	128	-	-	-	128	128
8	**13**	64	64	128	64	128	128
9	**14**	64	64	-	128	64	128
10	**15**	128	128	128	128	128	128
11	**20**	128	64	128	128	128	128
16	Gentamicin	-	-	-	0.5	1	0.5
17	Vancomycin	0.5	0.5	0.5	-	-	-

4.3 Evaluation of Anticancer Activity

The dichloromethane and acetone extracts of *Cassia alata* stems (SD and SA extracts, respectively) showed inactive anticancer against KB-oral carvity cancer, NCI-H187 small cell lung cancer, and MCF7-Breast cancer as showed in table 4-24. Therefore, no further evaluation anticancer activity of isolated compounds.

Table 4-24 Anticancer Activity of Acetone and Methanolic Extracts of *Cassia alata* Stems and Standard Markers

Sample	Anticancer activity (IC_{50}, μg/ml)		
	KB-oral carvity	NCI-H187	MCF7-Breast
Crude SD	inactive	inactive	inactive
Crude SA	inactive	inactive	inactive
Ellipticine	0.339	0.609	-
Doxorubicin	0.185	0.072	1.754

4.4 Evaluation of Antioxidation Activity

Evaluation of antioxidative effects has been carried out by various methods. The DPPH assay is one of the methods used for antioxidant testing on free radical terminator because its odd electron can be used as a convenient tool for the antioxidant assay. The DPPH free radical is dark violet solid, its solubility is not great, alcoholic solution having concentrations of approximately 5×10^{-4} are nevertheless densely colored. Its solution shows a strong absorption band at λ 517 nm (in ethanol). The capacity of the substances to donate electrons can be estimated from the degree of loss color (Blois, 1958). Coexistence of an antioxidant compound (AH) and free radical ($DPPH^{\bullet}$) leads to the disappearance of DPPH free radical and the appearance the free radical ($A^{\bullet}$) as shown in figure 4-1.

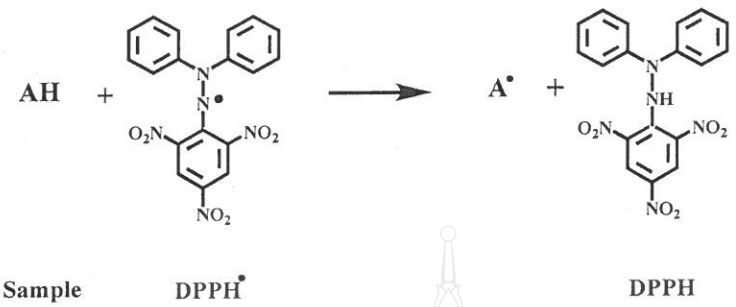


Figure 4-1 DPPH Free Radical and the Appearance of the Free Radical

4.4.1 Screening on the Free Radical Scavenging Activity and Evaluation of 50% Inhibition Concentration (IC_{50}) of Crude Extracts of *C. alata*

To determine the scavenging activity, the crude extracts of *C. alata* were tested for scavenging activity at the final concentration of 100 µg/mL. The activity was monitored by following the decrease of absorbance of the solution at 517 nm for 30 min, the stable capacity of the substances to donate electrons.

The results (table 4-25) presented that the crude LA is the most able to scavenge the DPPH radical followed by crude TA and RA extracts with percentage inhibition of 89.65 ± 0.15, 80.65 ± 0.20 and 80.49 ± 0.08, respectively which were effective than BHT (69.45 ± 0.18 % inhibition). Whereas the extracts SA and FA showed percentage of scavenge more than 50% at 30 min (64.18 ± 0.18 and 56.18 ± 0.09 % inhibition, respectively). The other crude extracts (SD, LD, FM, TD and FD) inhibited percentage of inhibition less than 50% at 30 min. The results were expressed as % inhibition as shown in table 4-25. The average absorption and % inhibition of FA, LA, RA, SA, and TA crude extracts at various final concentrations were at 100.00, 98.36, 49.18, 24.59, 12.30, 6.15, 3.08, and 1.54 µg/mL. Their IC_{50} were shown at 85.25 ± 0.06, 41.80 ± 0.07, 29.51 ± 0.12, 59.02 ± 0.11, and 26.23 ± 0.09 µg/mL, respectively. Crude LA, RA, SA, and TA extracts showed moderately antioxidative activity.

Table 4-25 The % Inhibition and IC_{50} Values of *C. alata* Crude Extracts

Sample	% inhibition (at 30 min) Final Conc. of 100 µg/mL	IC_{50} (µg/mL, 30 min) Final Conc. of 100 µg/mL
Crude FD	14.26 ± 0.11	-
Crude FA	56.18 ± 0.09	85.25 ± 0.06
Crude FM	30.83 ± 0.06	-
Crude LD	30.98 ± 0.13	-
Crude LA	89.65 ± 0.15	41.80 ± 0.07
Crude RA	80.49 ± 0.08	29.51 ± 0.12
Crude SD	38.39 ± 0.11	-
Crude SA	64.18 ± 0.18	59.02 ± 0.11
Crude TD	30.78 ± 0.13	-
Crude TA	80.65 ± 0.20	26.23 ± 0.09
Ascorbic acid	97.21 ± 0.25	2.79 ± 0.15
BHT	69.45 ± 0.18	12.30 ± 0.12

4.4.2 Screening on the Free Radical Scavenging Activity and Evaluation of 50% Inhibition Concentration (IC_{50}) of Pure Compounds of *C. alata*

The pure compounds of *C. alata* were tested for scavenging activity at the final concentration of 50 µM. The activity was monitored by following the decrease of absorbance of the solution at 517 nm for 30 min, the stable capacity of the substances to donate electrons.

The screening on the free radical scavenging results (table 4-26) indicated that compounds **7** and **14** are the most able to scavenge the DPPH radical with 79.00 ± 0.19 and 55.68 ± 0.22 % inhibition, respectively. Whereas compounds **2**, **22**, **13**, **9**, **5**, **15**, and **4** showed the percentage of inhibition less than 50% at 30 min. The average absorption and % inhibition of compounds **7** and **14** at various final concentrations (50.00, 40.98, 37.54, 32.79, 25.08, 24.59, 12.46, 6.23, 3.11, and 1.56 µM) were expressed at IC_{50} values of 9.67 ± 0.29 and 45.90 ± 0.22 µM, respectively (table 4-26). Compound **7** showed stronger antioxidative activity (IC_{50} 9.67 ± 0.29 µM) than

ascorbic acid (IC_{50} 25.41 ± 0.92 μM). Compound **14** showed moderate antioxidative activity (IC_{50} 45.90 ± 0.22 μM) which was more effective than BHT (IC_{50} 45.56 ± 0.45 μM).

Table 4-26 The % Inhibition and IC_{50} Values of Isolated Pure Compounds from *C. alata* at Final Concentration of 50 μM

Sample	% inhibition (at 30 min)	IC_{50} (μM, 30 min)
2	24.67 ± 0.18	-
4	1.20 ± 0.10	-
5	1.66 ± 0.15	-
7	79.00 ± 0.19	9.67 ± 0.29
9	4.32 ± 0.17	-
13	5.18 ± 0.20	-
14	55.68 ± 0.22	45.90 ± 0.22
15	1.56 ± 0.11	-
22	7.54 ± 0.15	-
Ascorbic acid	88.34 ± 0.32	25.41 ± 0.92
BHT	52.86 ± 0.19	46.56 ± 0.45

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3. Publications

Kulsiri Yossathera, Sarin Sriprang, Siripat Suteerapataranon, and Suwanna Deachathai, 2014. "Antibacterial and antioxidative compounds from *Oroxylum indicum*(L.)Vent." Chemistry of Natural Compounds (in press).

Trinop Promgool, Orasa Pancharoen, and Suwanna Deachathai, 2014. "Antibacterial and antioxidative compounds from *Cassia alata* Linn." Songklanakarin Journal of Science and Technology (in press).

Orawan Suwantong, Porntipa Pankongadisak, Suwanna Deachathai, and Pitt Supaphol, 2014. "Electrospun poly(L-lactic acid) fiber mats containing crude *Garcinia mangostana* extract for use as wound dressing" Polym Bull., DOI 10.1007/s00289-014-1102-9.

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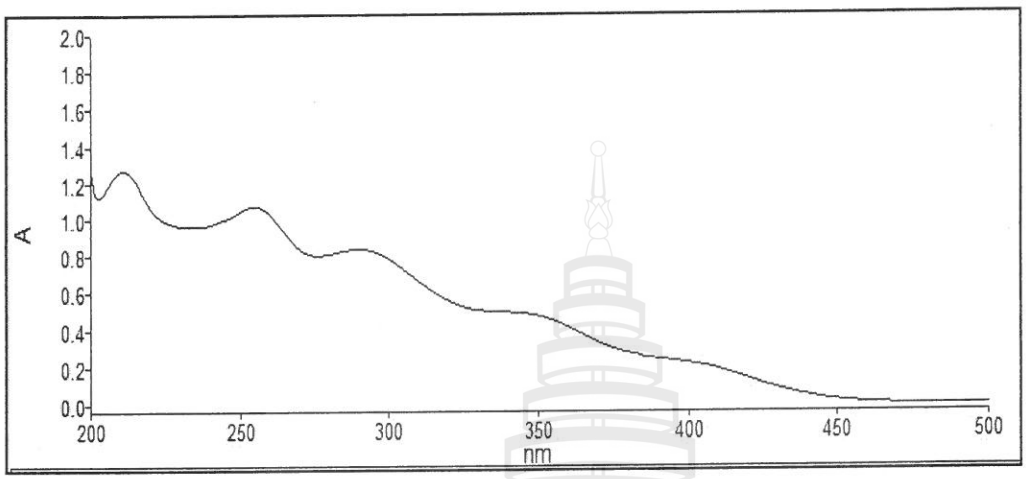
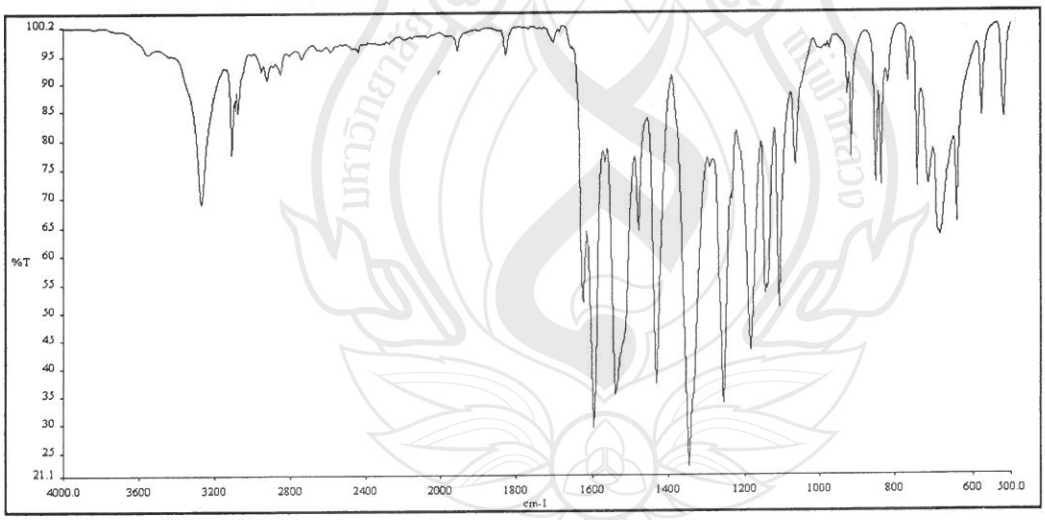
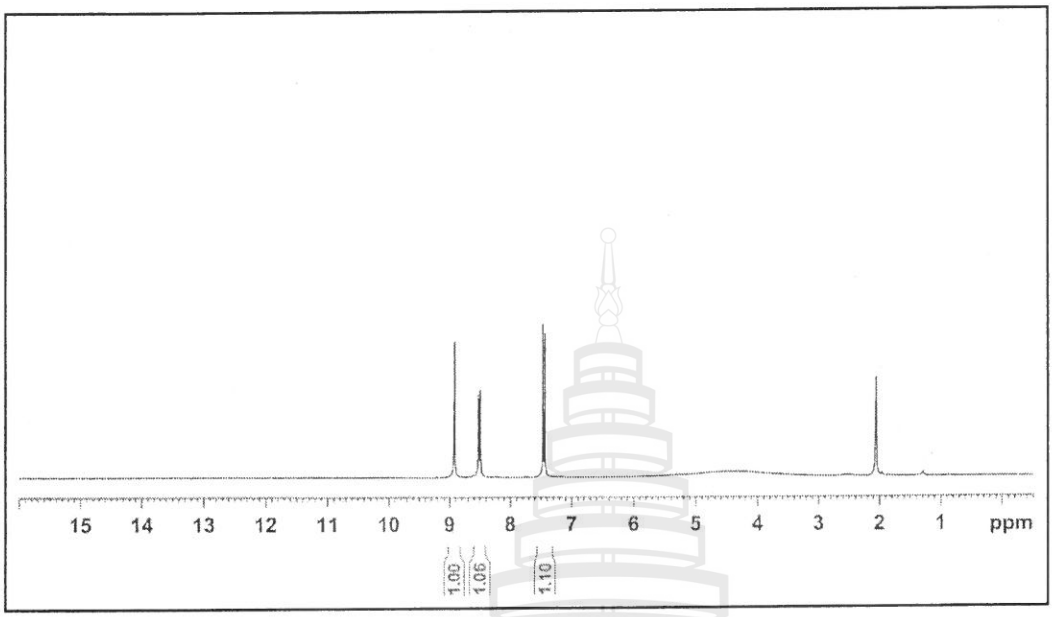
APPENDIX



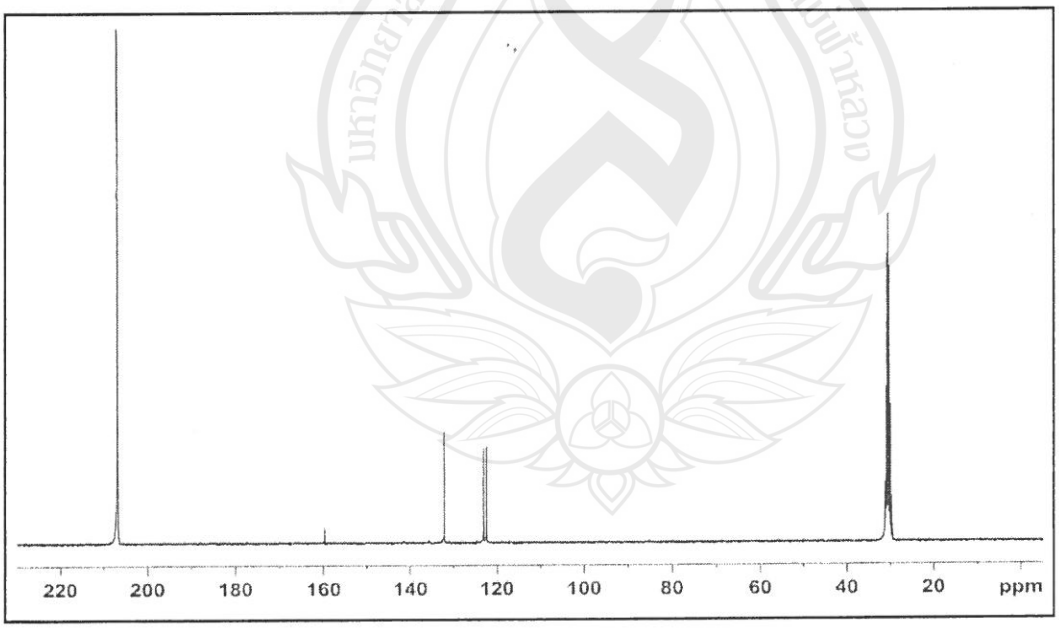
UV (MeOH) spectrum of **1**



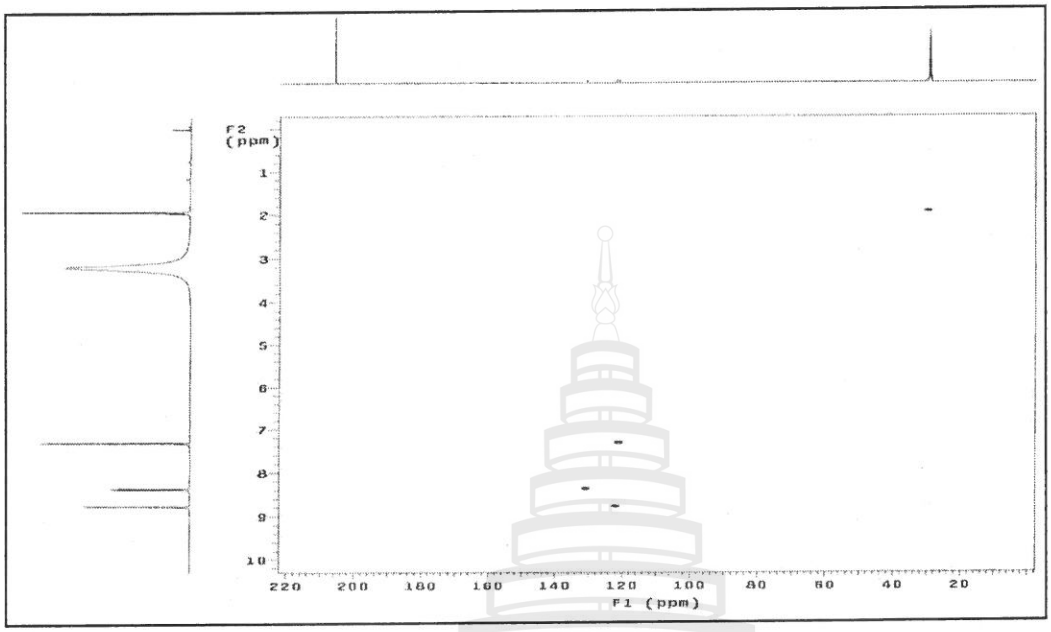
IR (KBr) spectrum of **1**



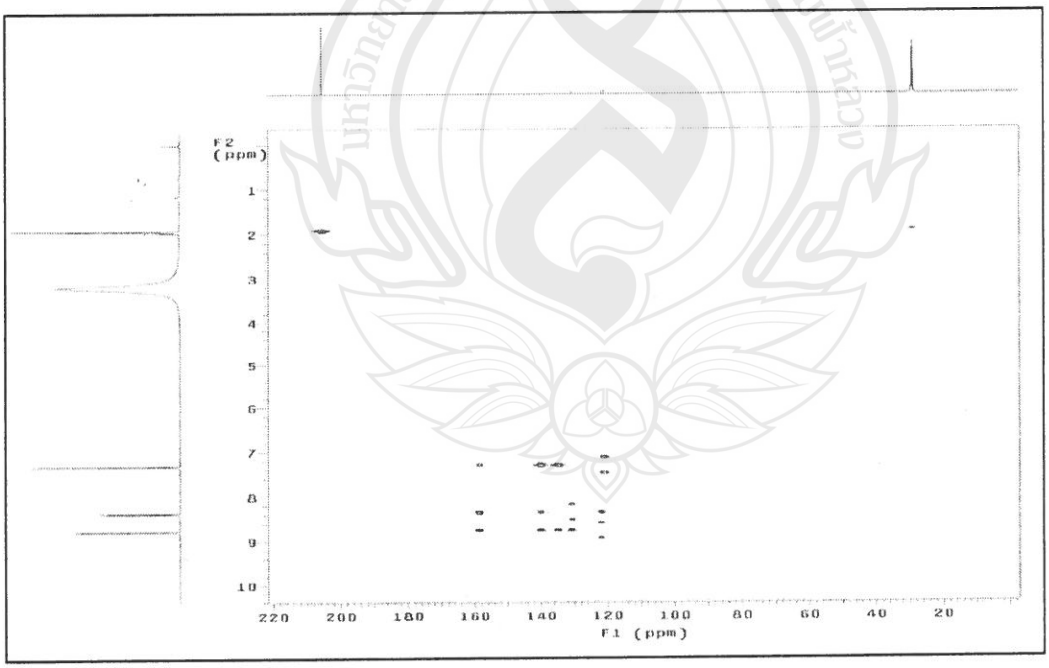
^{1}H NMR (300 MHz, acetone-d_6) spectrum of **1**



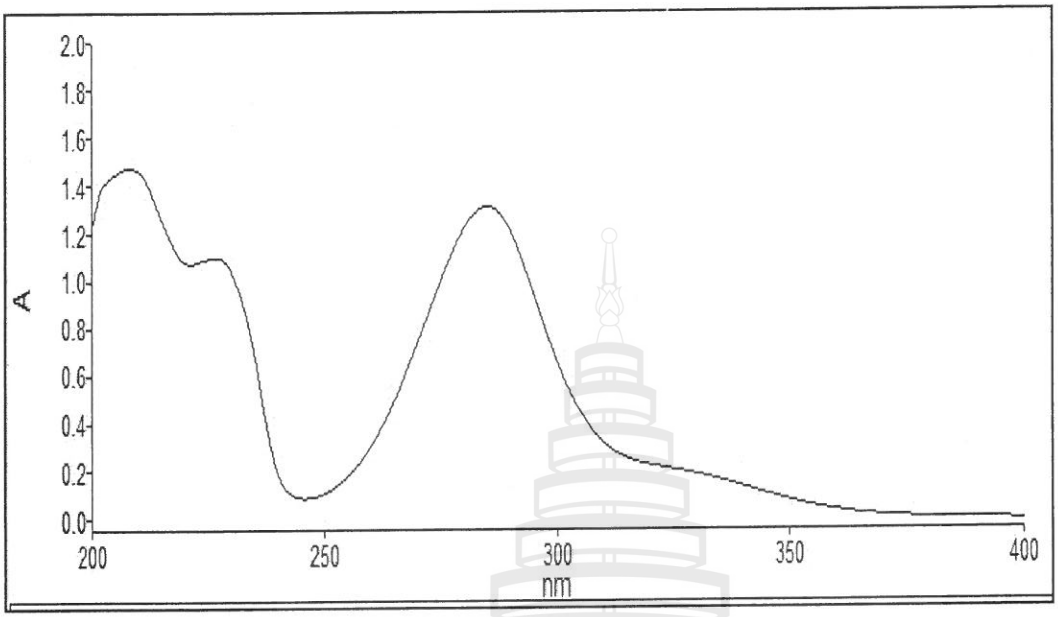
^{13}C NMR (75 MHz, acetone-d_6) spectrum of **1**



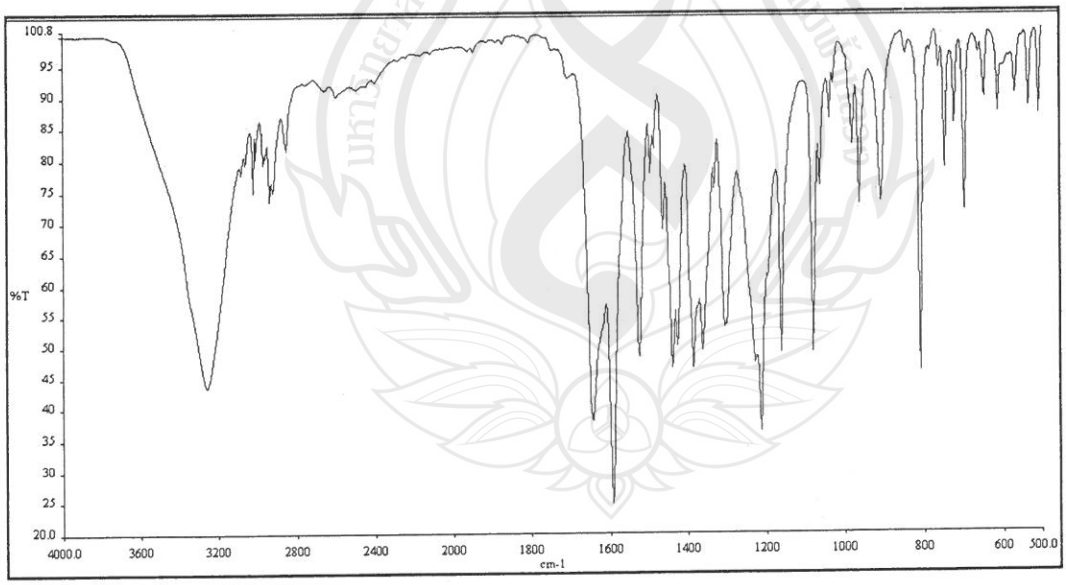
HMQC (acetone-d_6)spectrum of **1**



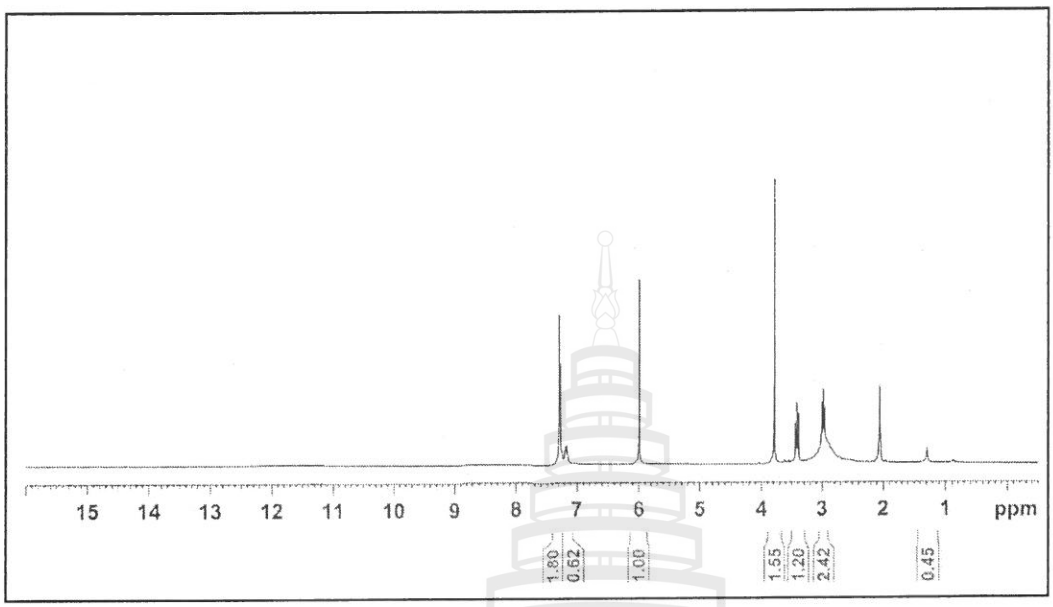
HMBC (acetone-d_6) spectrum of **1**



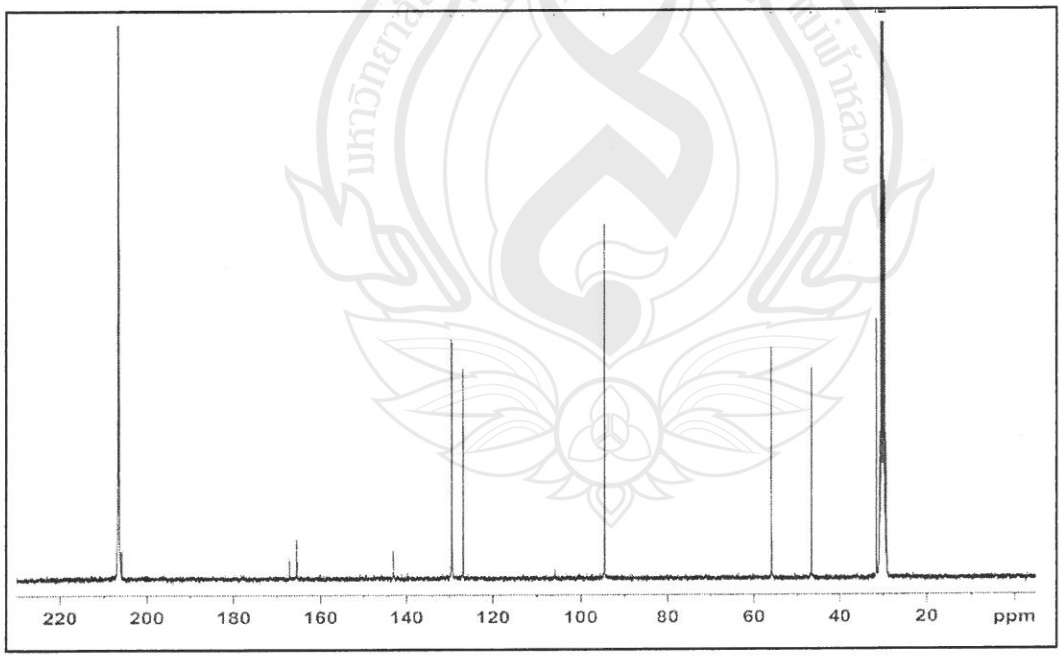
UV (MeOH) spectrum of **2**



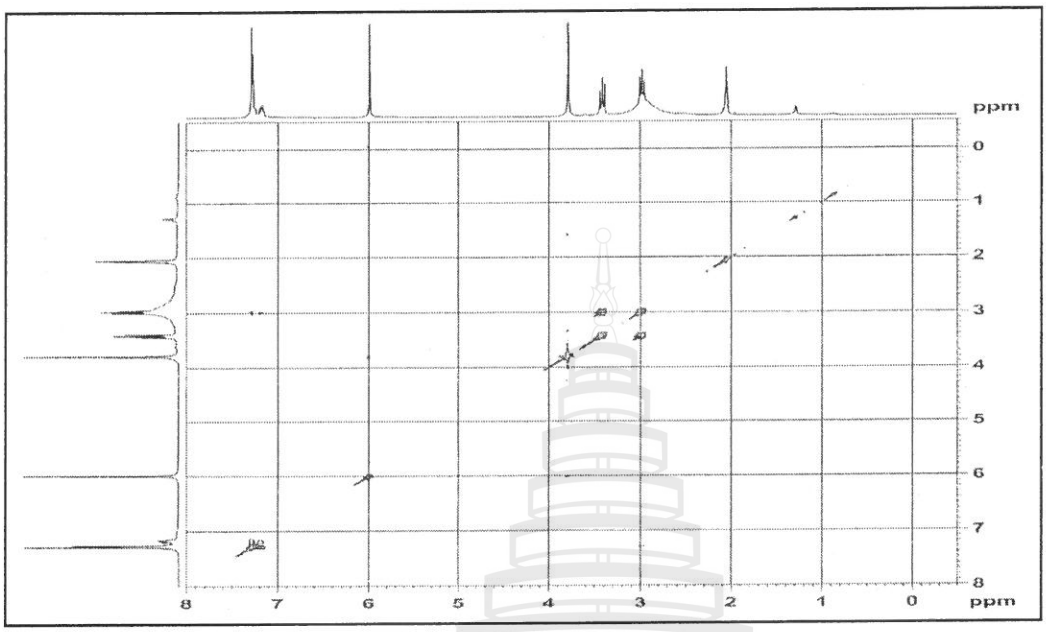
IR (KBr) spectrum of **2**



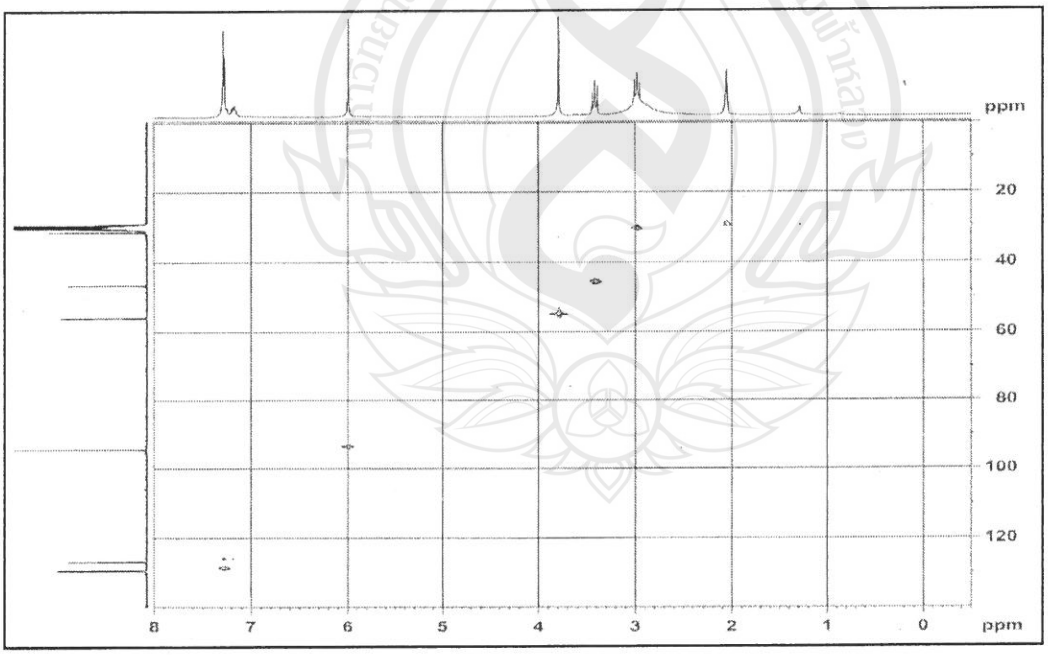
^{1}H NMR (300 MHz, acetone-d_6) spectrum of **2**



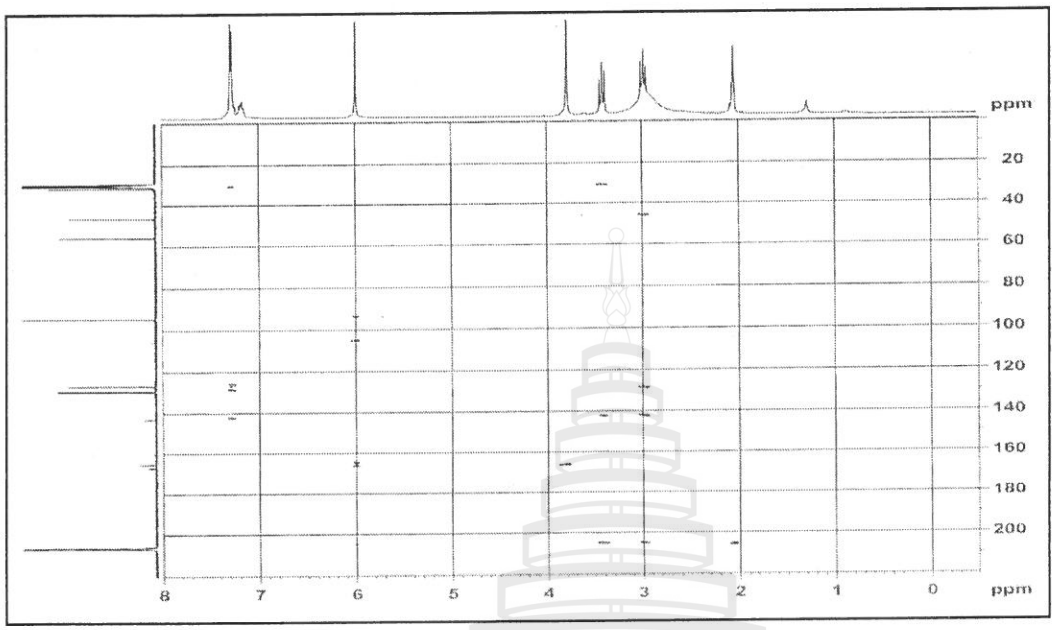
^{13}C NMR (75 MHz, acetone-d_6) spectrum of **2**



COSY (acetone-d_6) spectrum of **2**

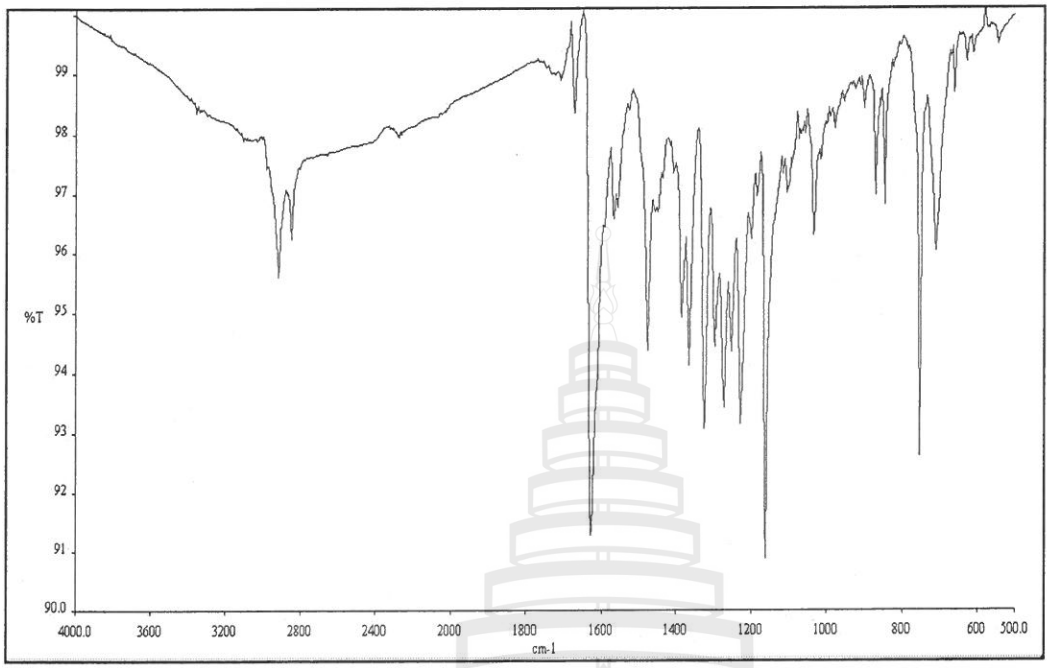


HMQC (acetone-d_6) spectrum of **2**

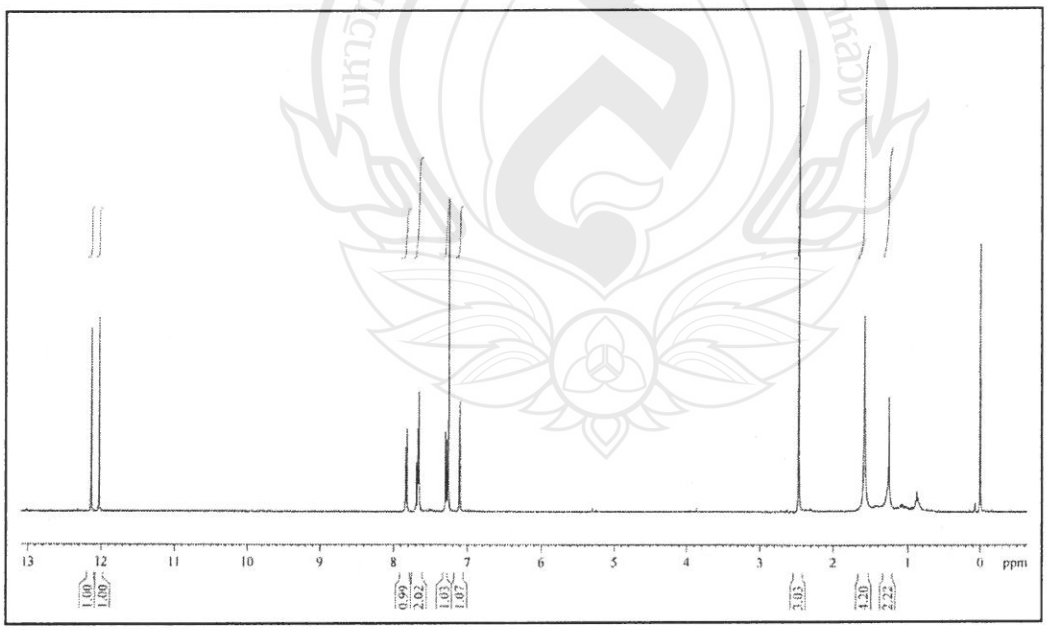


HMBC (acetone-d_6) spectrum of **2**

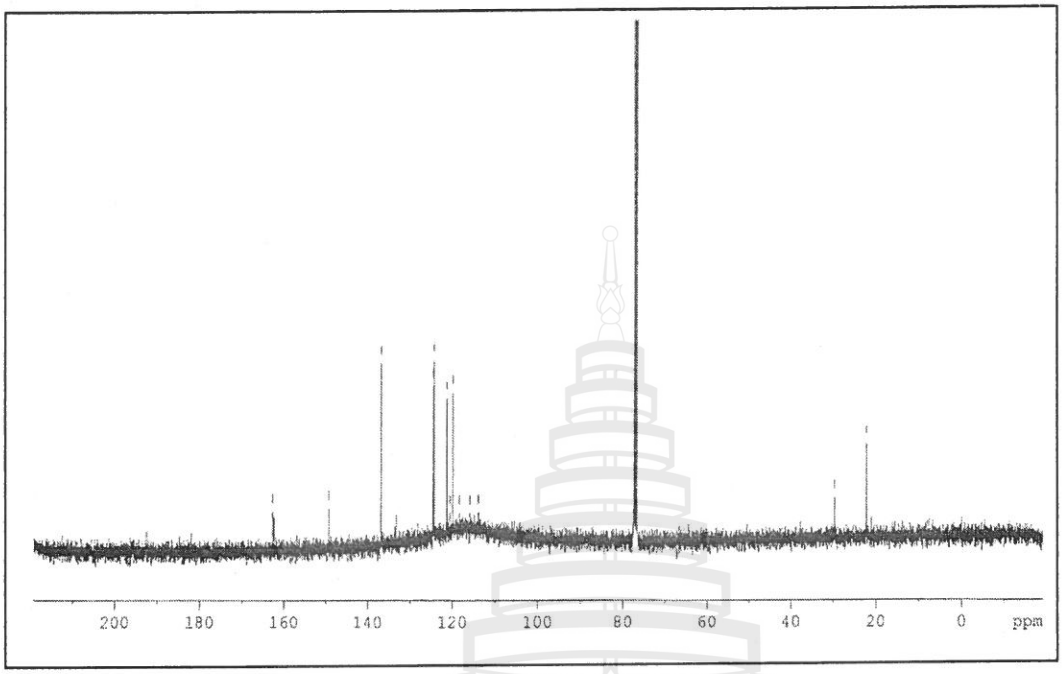
UV (MeOH) spectrum of **4**



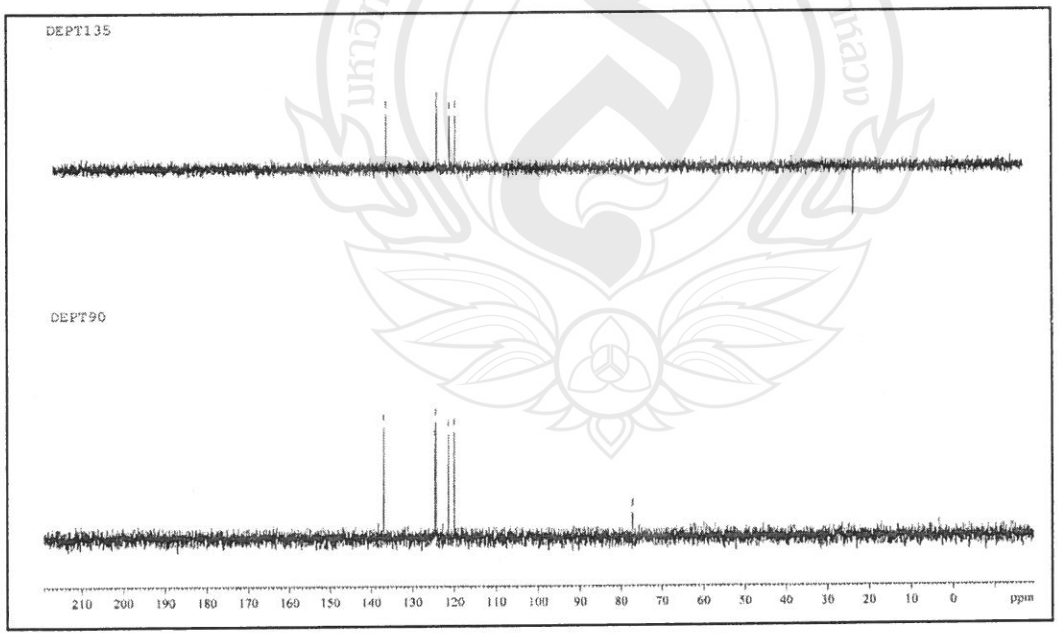
IR (KBr) spectrum of **4**



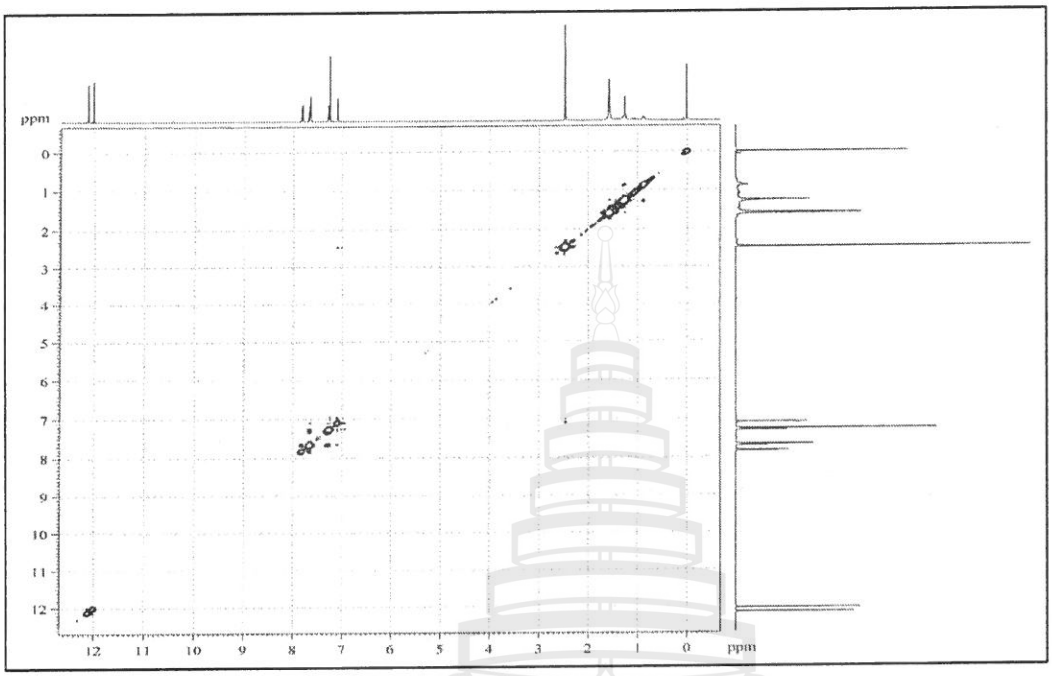
^{1}H NMR (400 MHz, acetone-d_6) spectrum of **4**



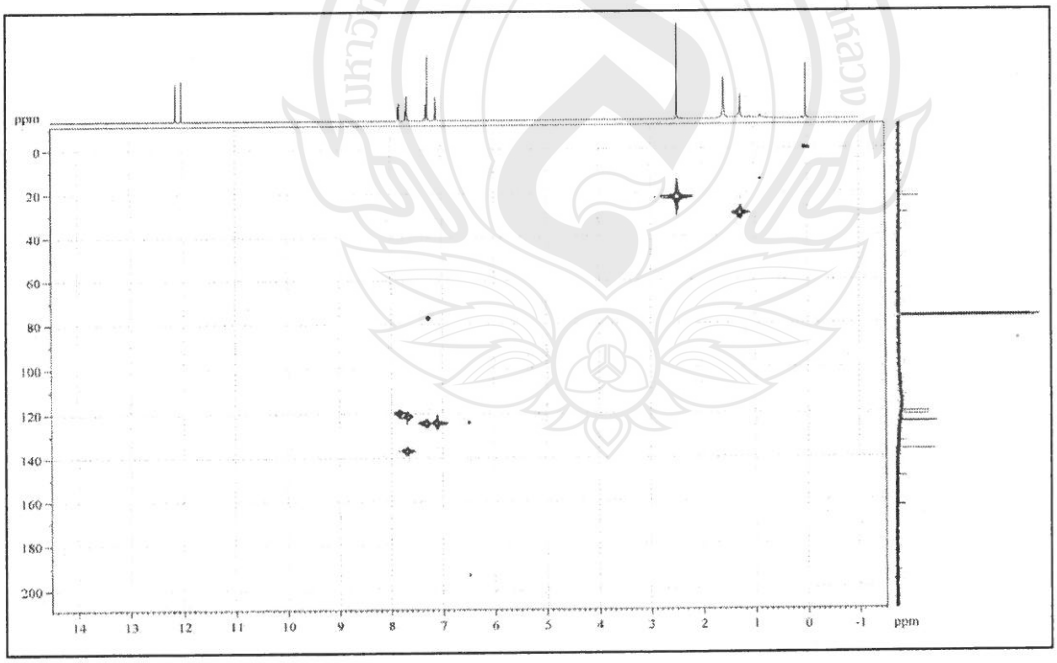
^{13}C NMR (100 MHz, acetone-d_6) spectrum of **4**



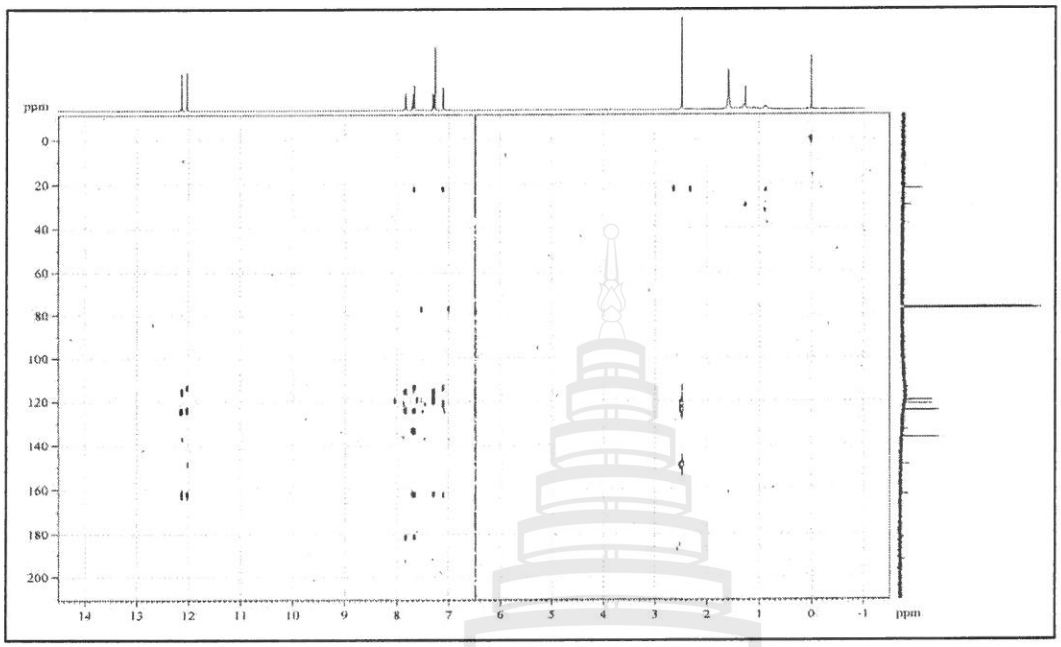
DEPT 135° and 90°(acetone-d_6) spectrum of **4**



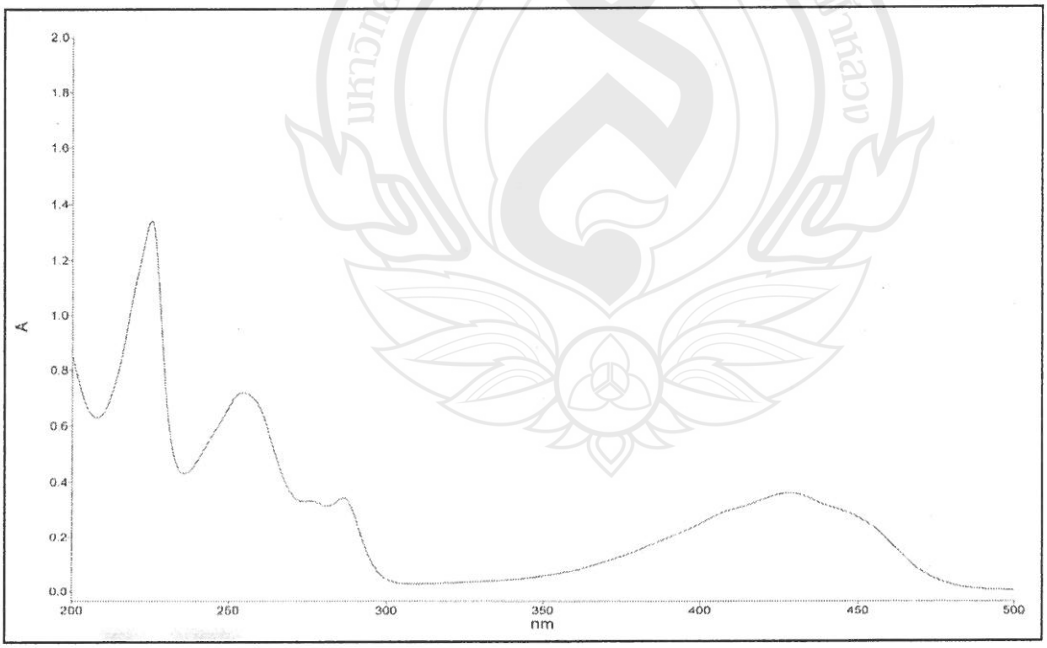
COSY (acetone-d_6) spectrum of **4**



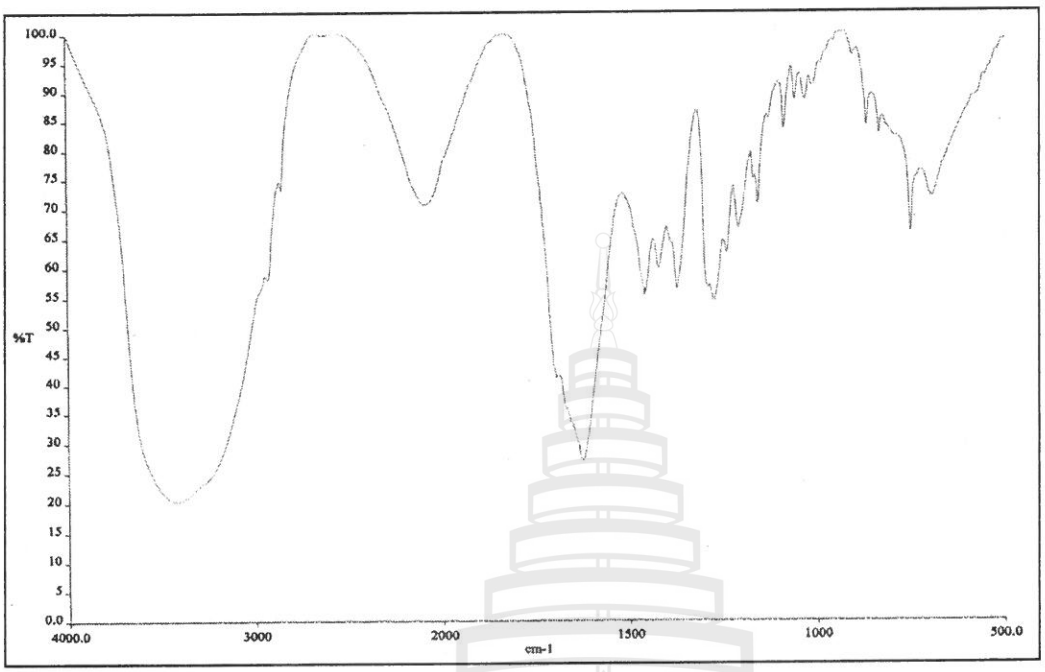
HMQC (acetone-d_6) spectrum of **4**



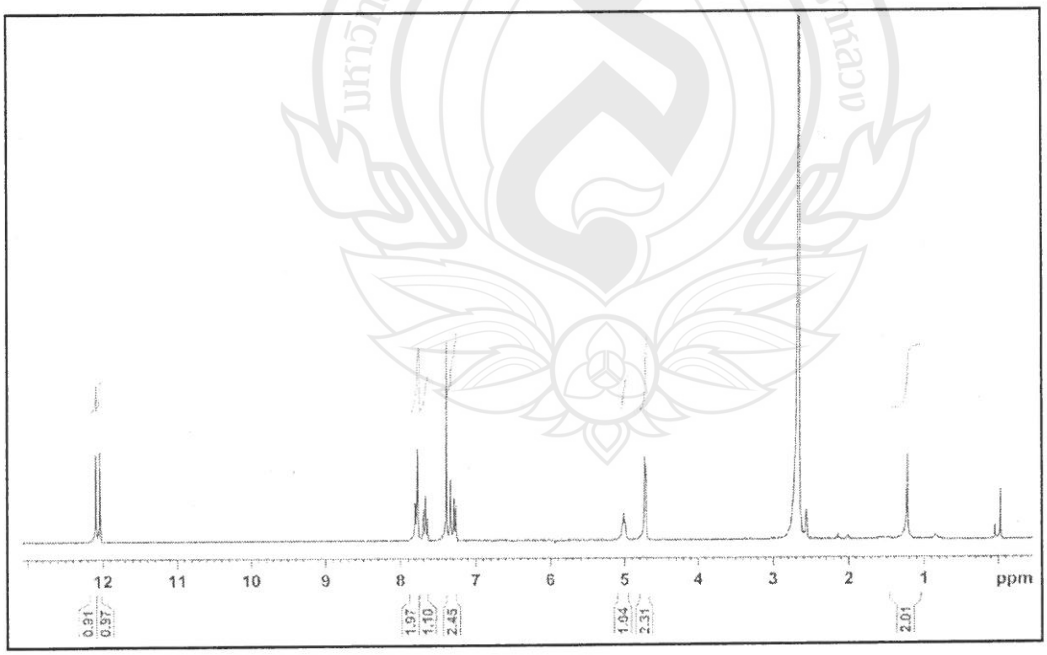
HMBC (acetone-d_6) spectrum of **4**



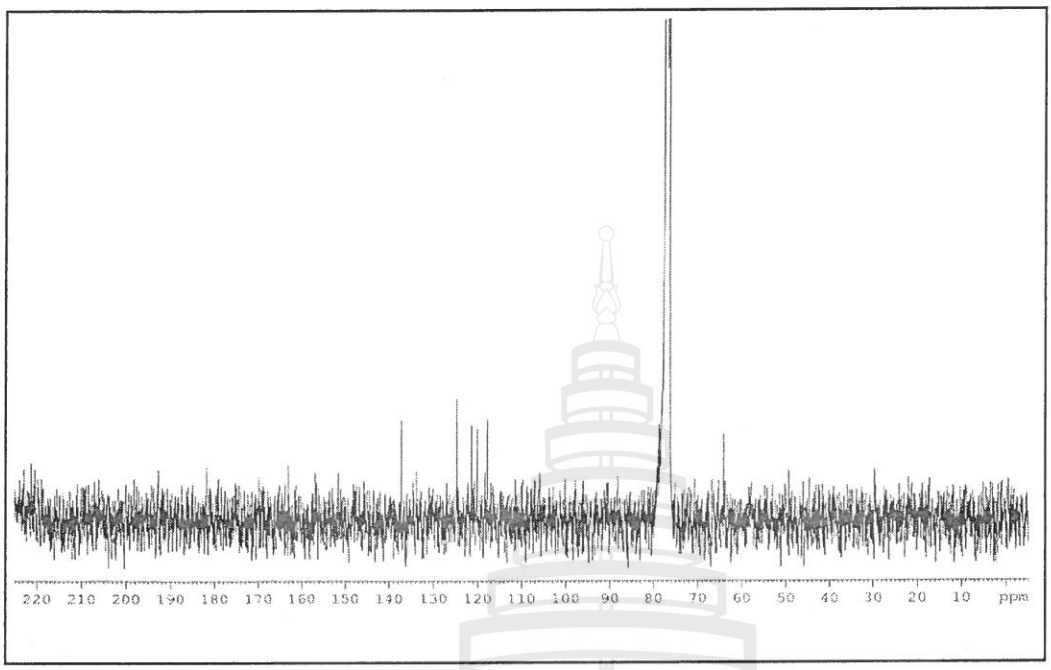
UV (MeOH) spectrum of **5**



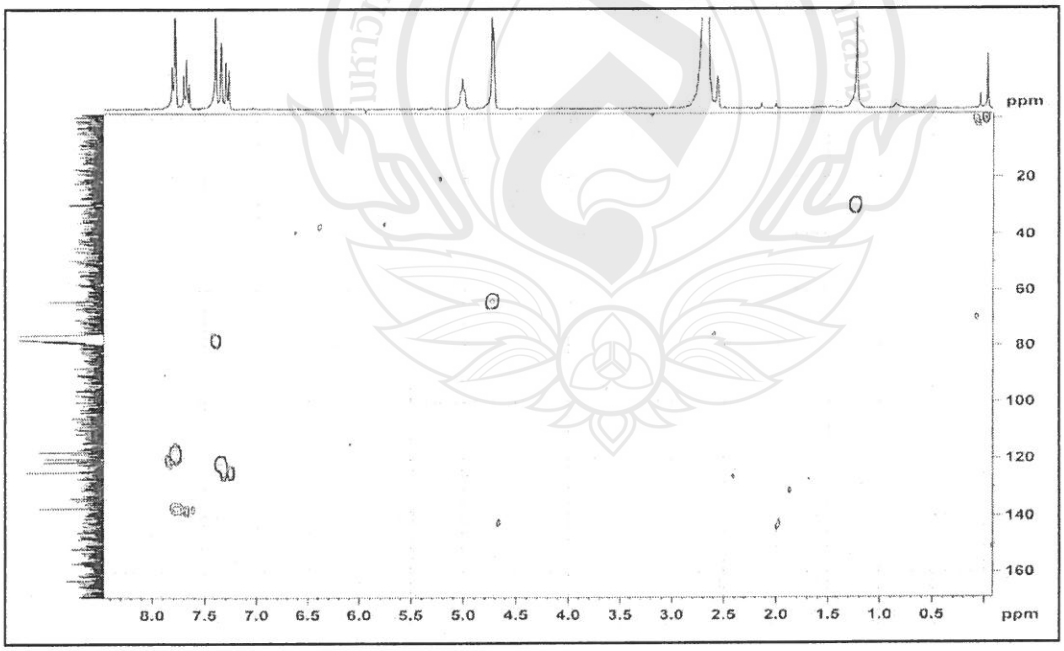
IR (KBr) spectrum of **5**



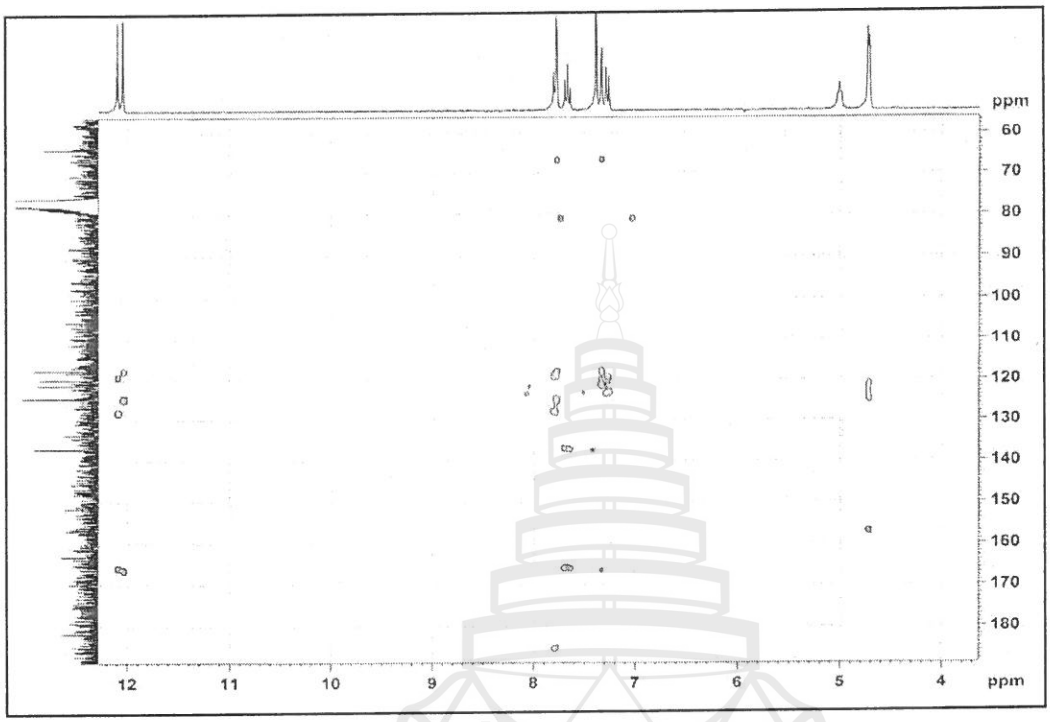
^{1}H NMR (300 MHz, $CDCl_3$+DMSO-d_6) spectrum of **5**



^{13}C NMR (75 MHz, $CDCl_3$+DMSO-d_6) spectrum of **5**

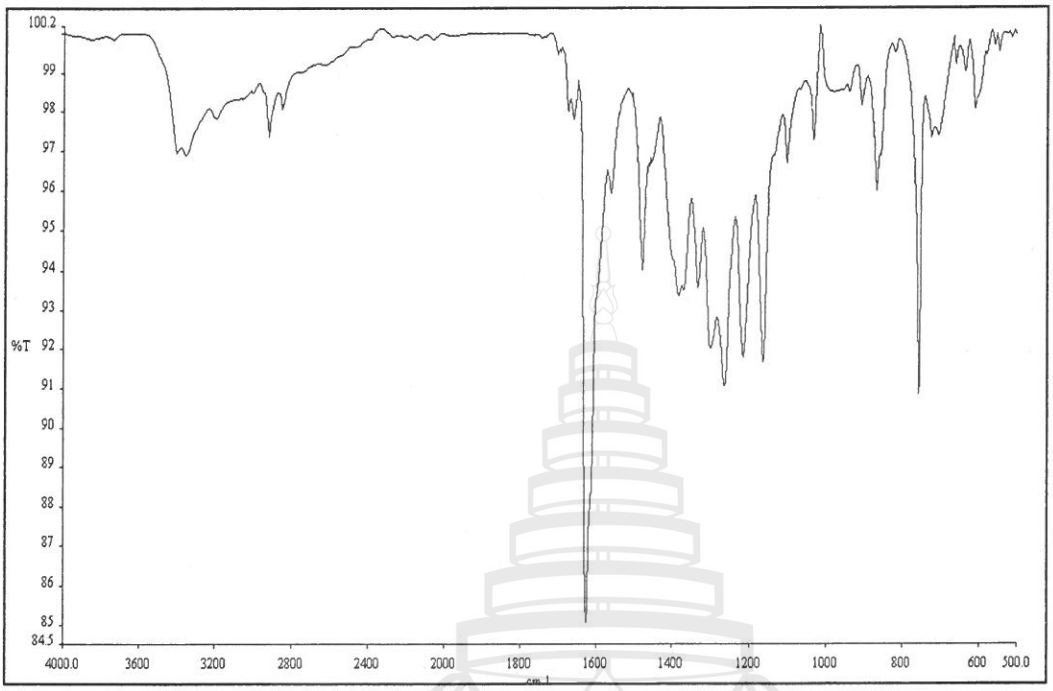


HMQC ($CDCl_3$+DMSO-d_6) spectrum of **5**



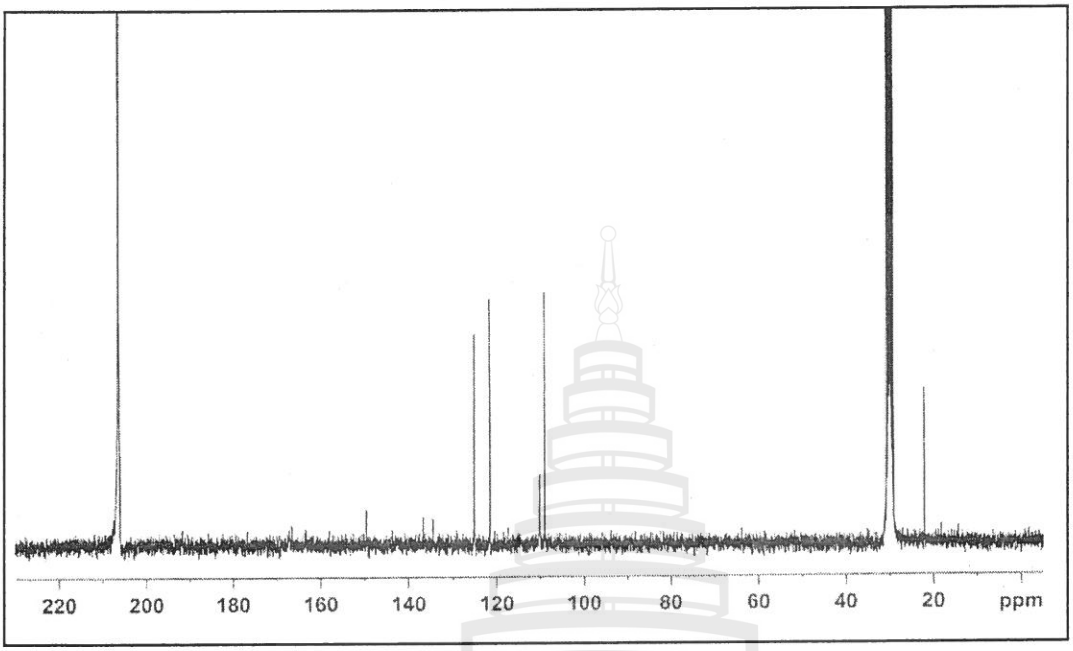
HMBC ($CDCl_3$+DMSO-d_6) spectrum of **5**

UV (MeOH) spectrum of **6**

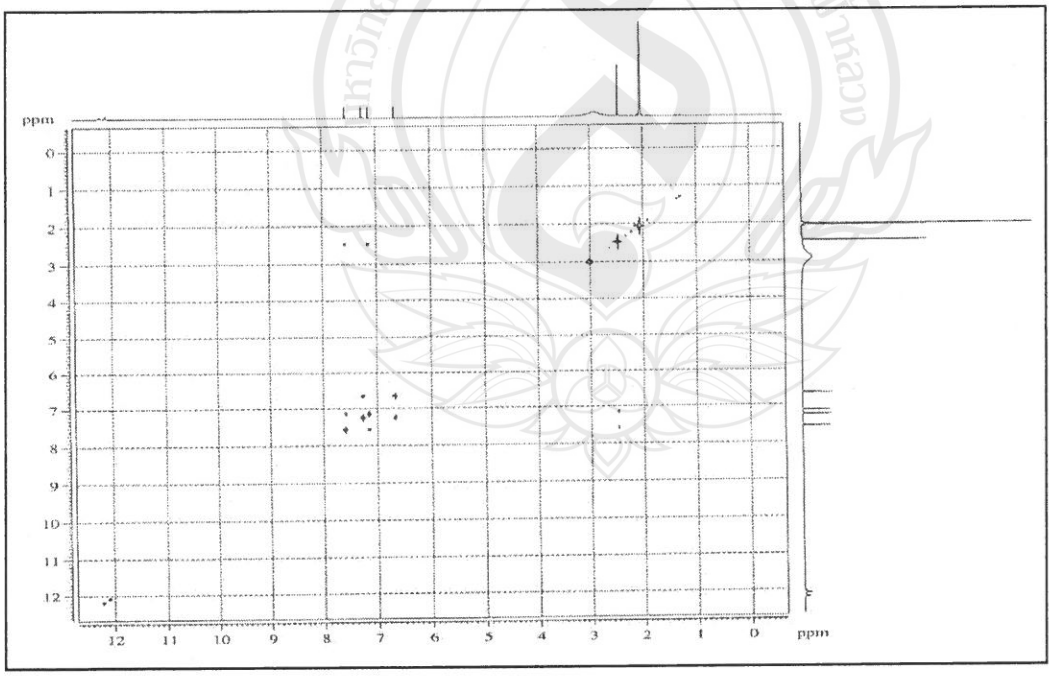


IR (KBr) spectrum of **6**

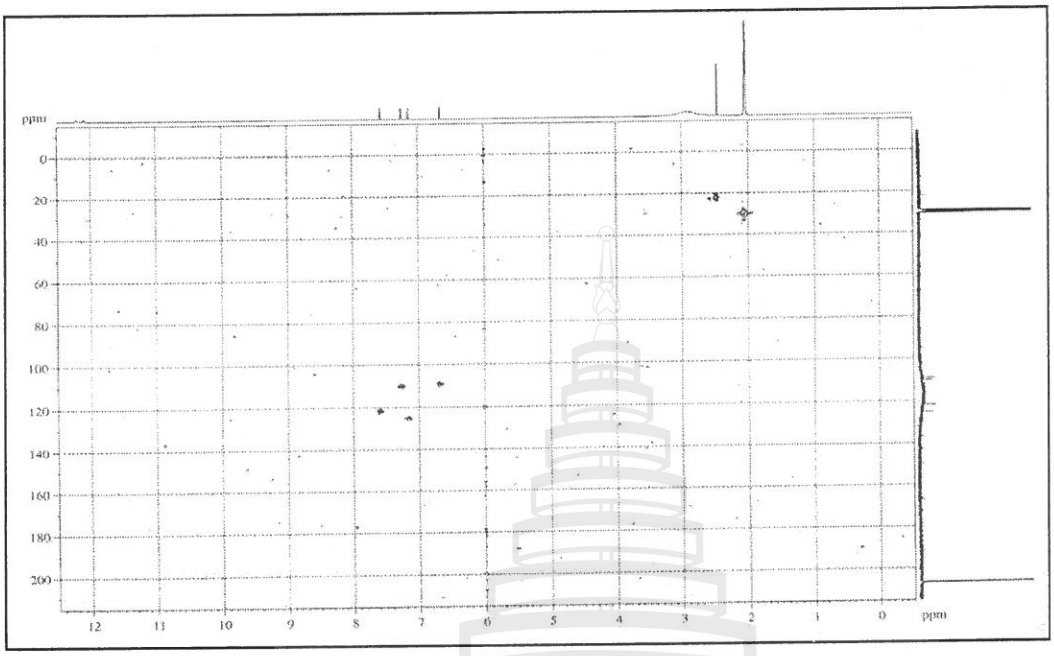
^{1}H NMR (400 MHz, acetone-d_6) spectrum of **6**



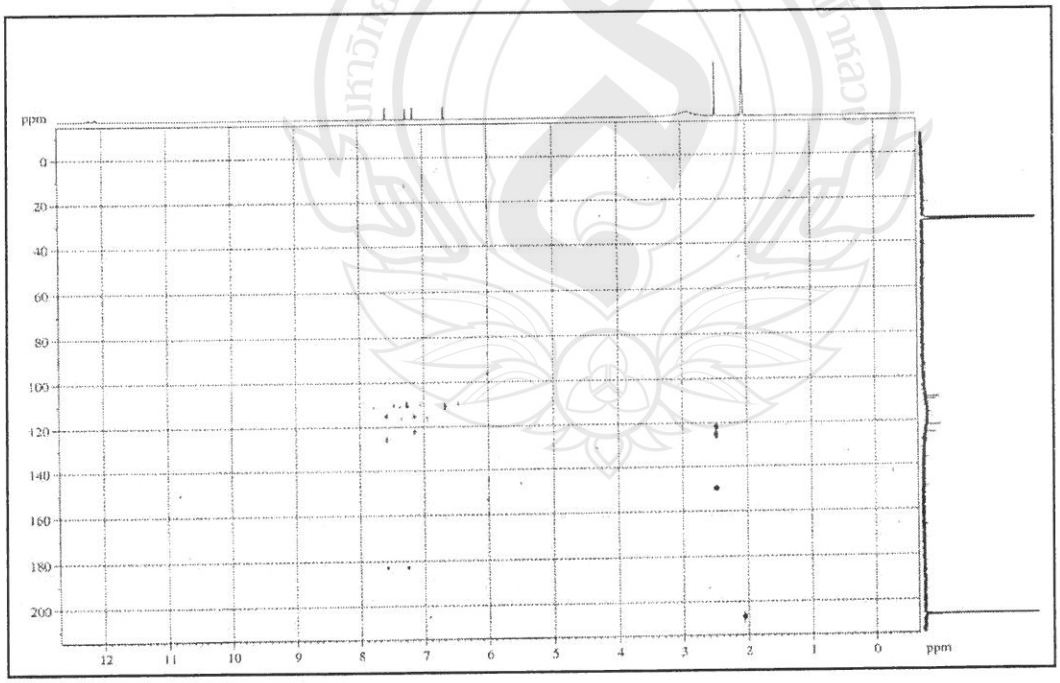
^{13}C NMR (100 MHz, acetone-d_6) spectrum of **6**



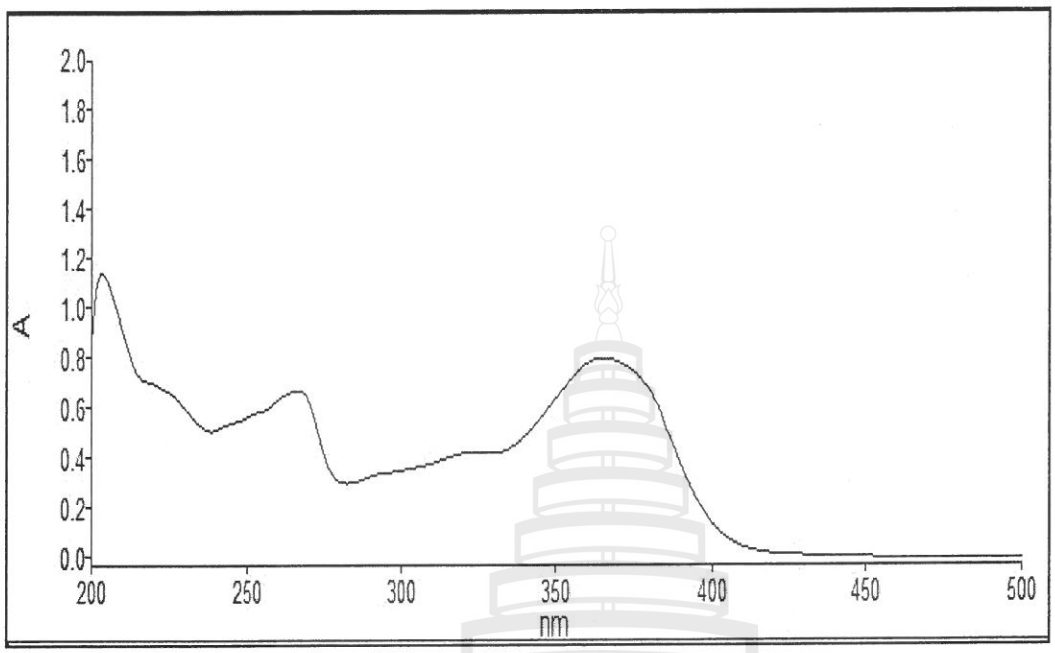
COSY (acetone-d_6) spectrum of **6**



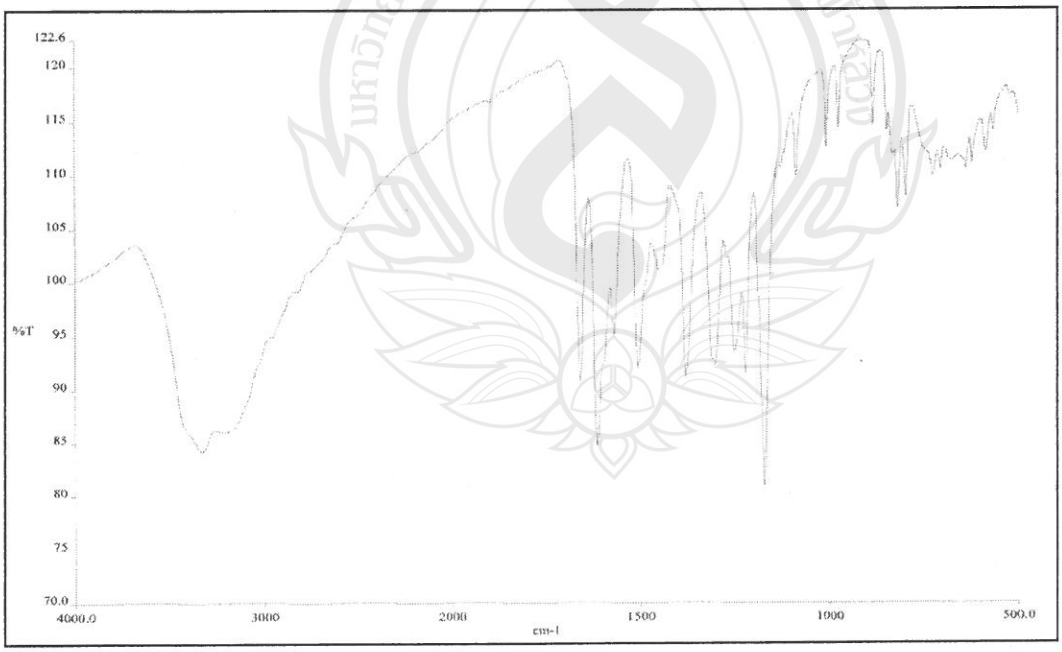
HMQC (acetone-d_6) spectrum of **6**



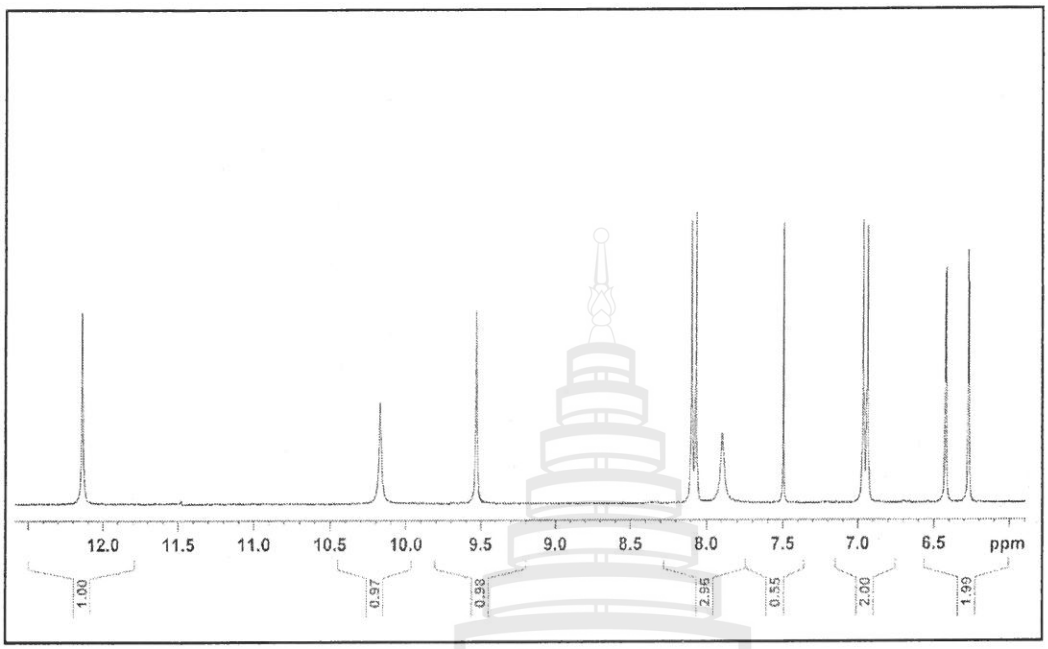
HMBC (acetone-d_6) spectrum of **6**



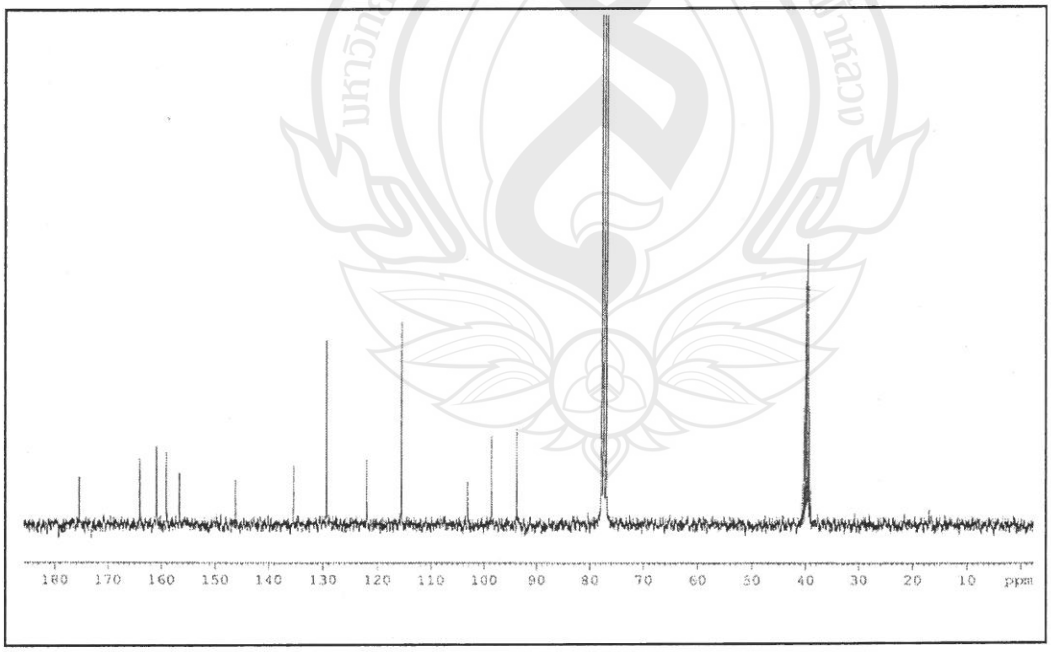
UV (MeOH) spectrum of 7



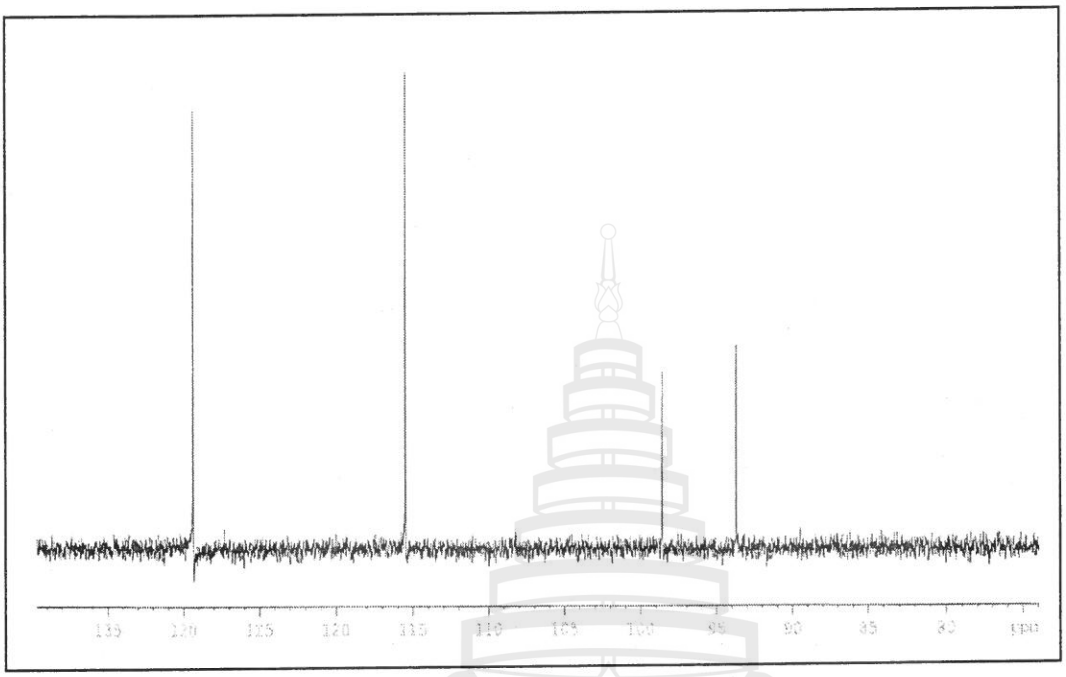
IR (KBr) spectrum of 7



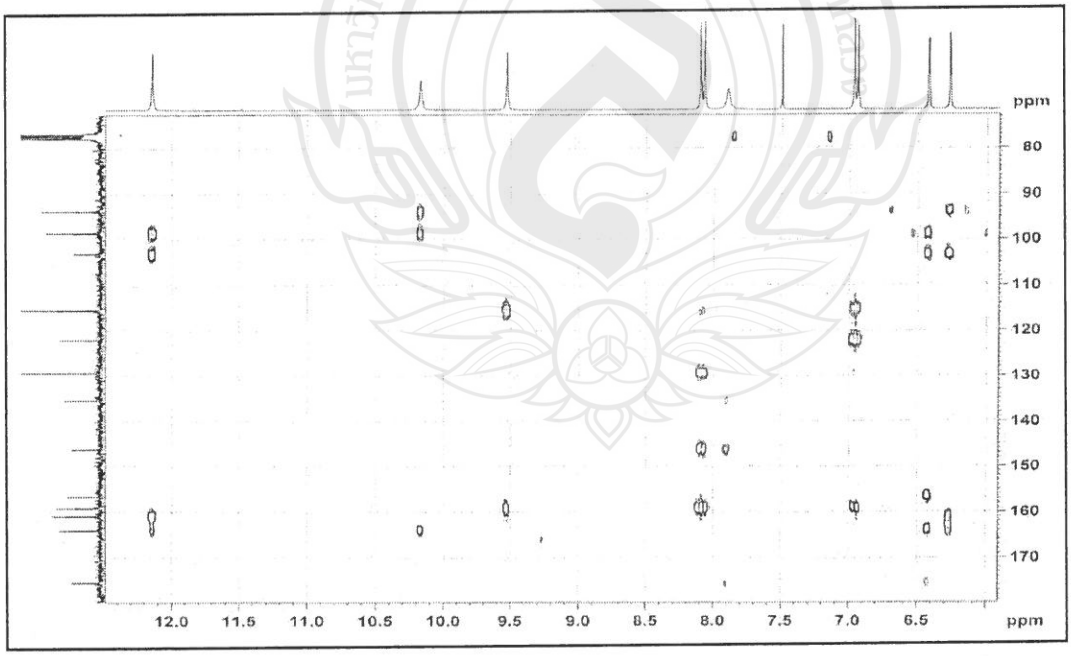
^{1}H NMR (300 MHz, acetone-d_6) spectrum of **7**



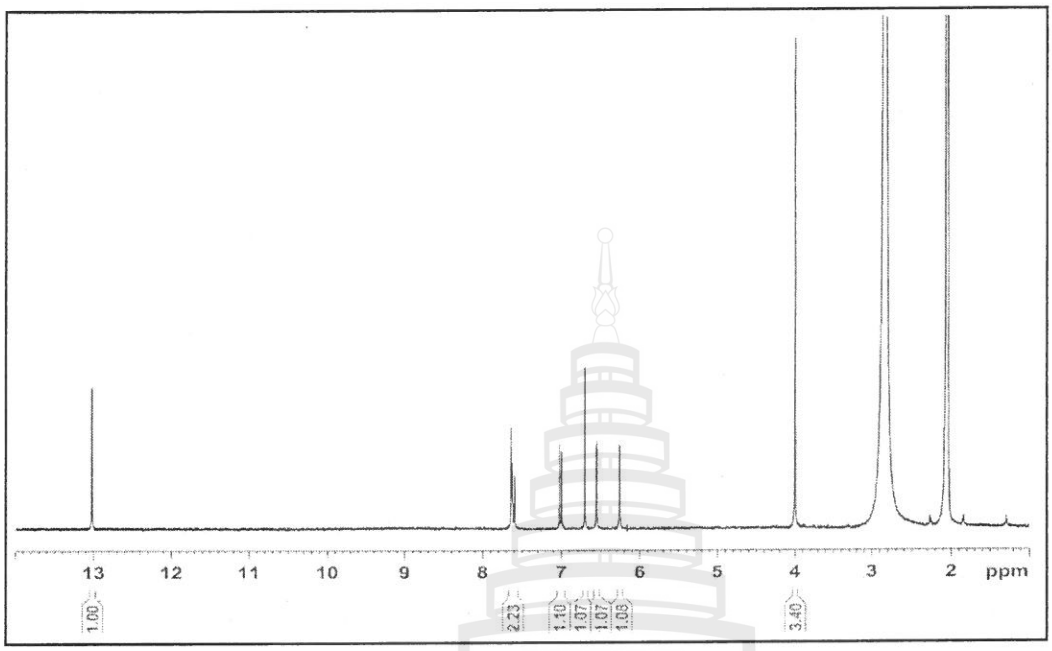
^{13}C NMR (75 MHz, acetone-d_6) spectrum of **7**



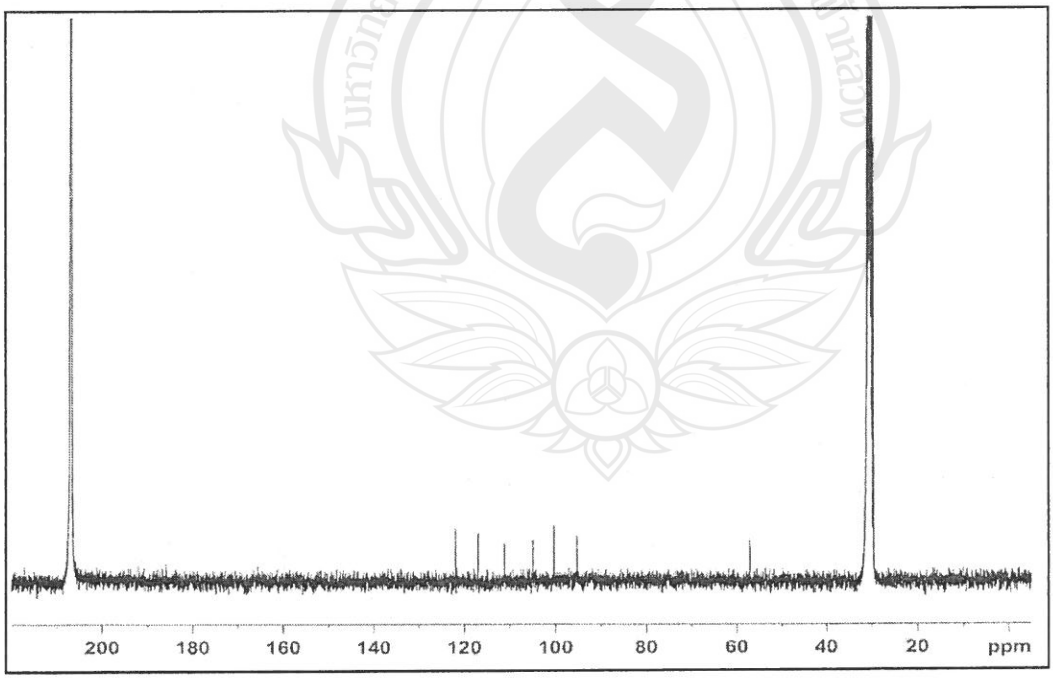
DEPT 90° (acetone-d_6) spectrum of **7**



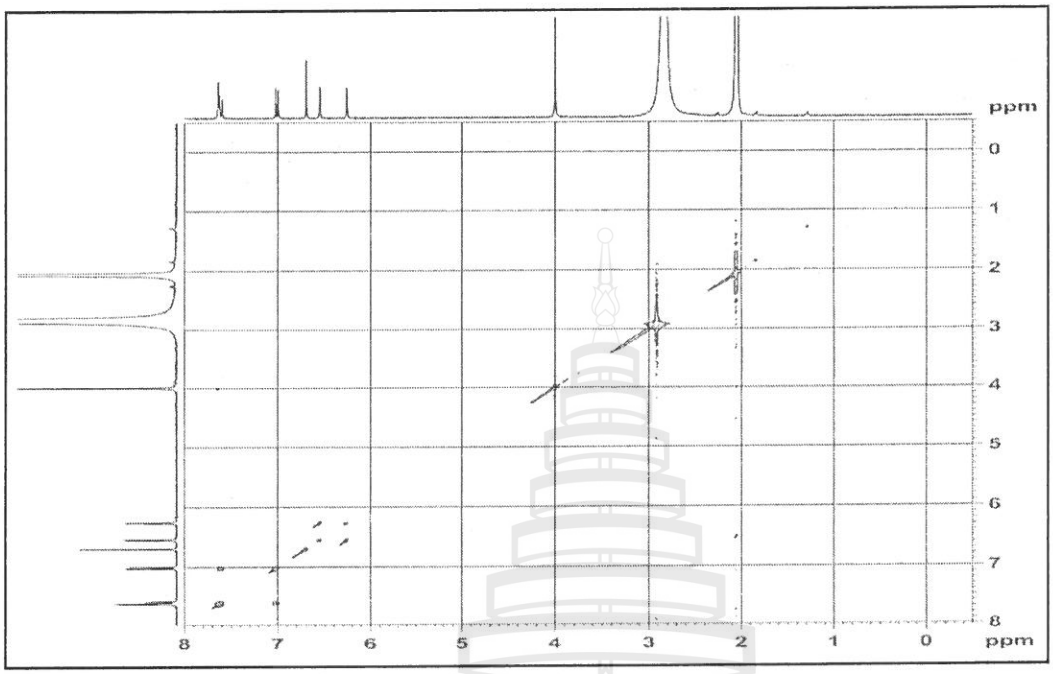
HMBC (acetone-d_6) spectrum of **7**



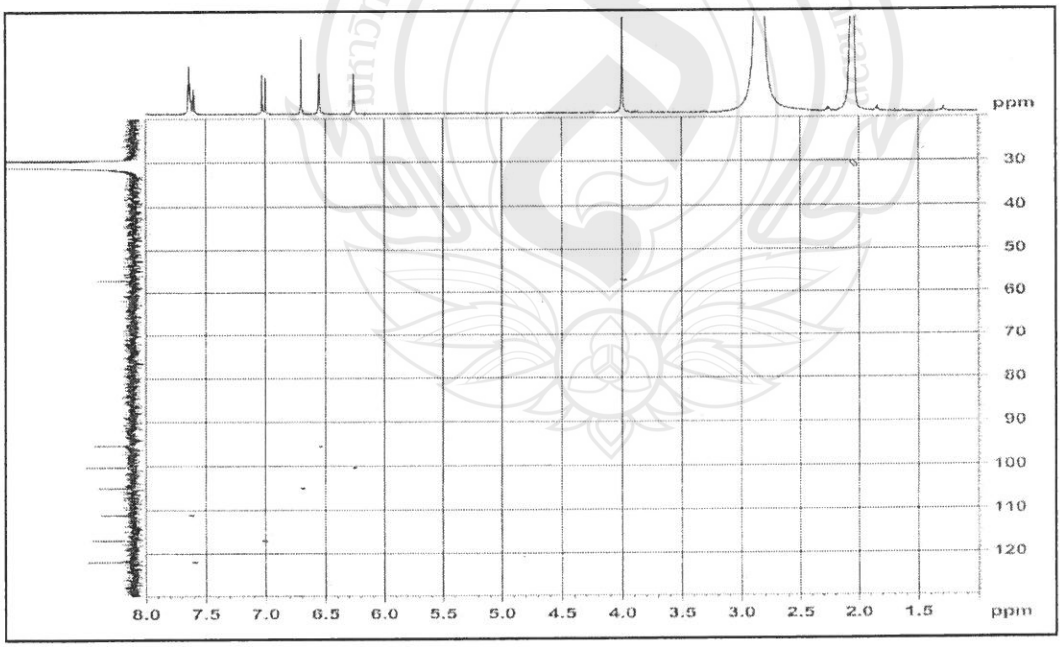
^{1}H NMR (300 MHz, acetone-d_6) spectrum of **8**



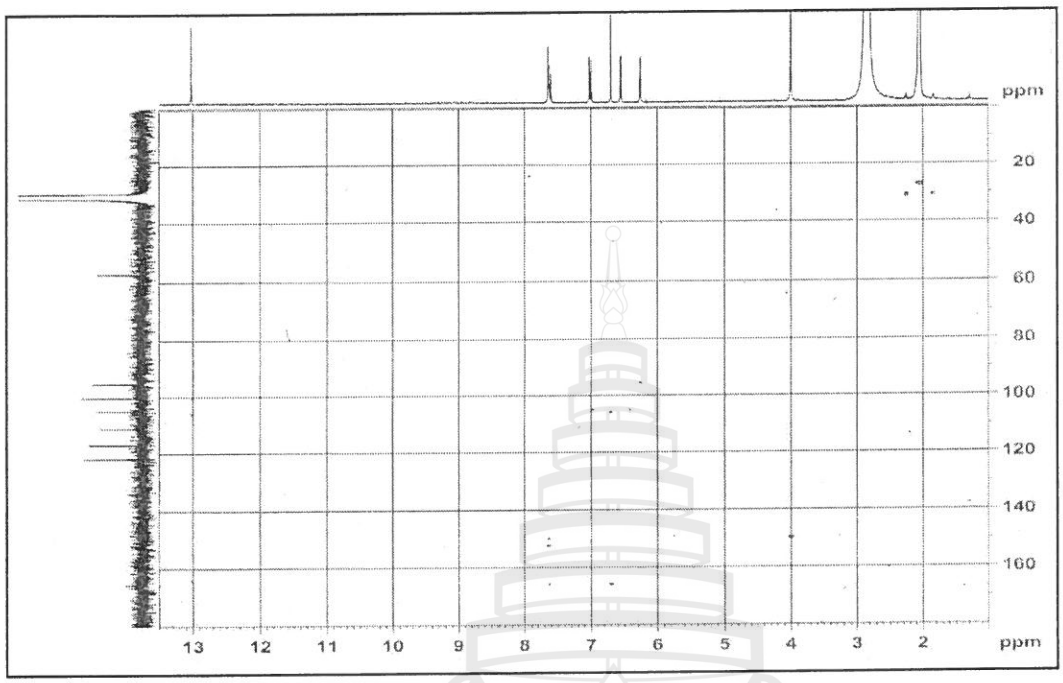
^{13}C NMR (75 MHz, acetone-d_6) spectrum of **8**



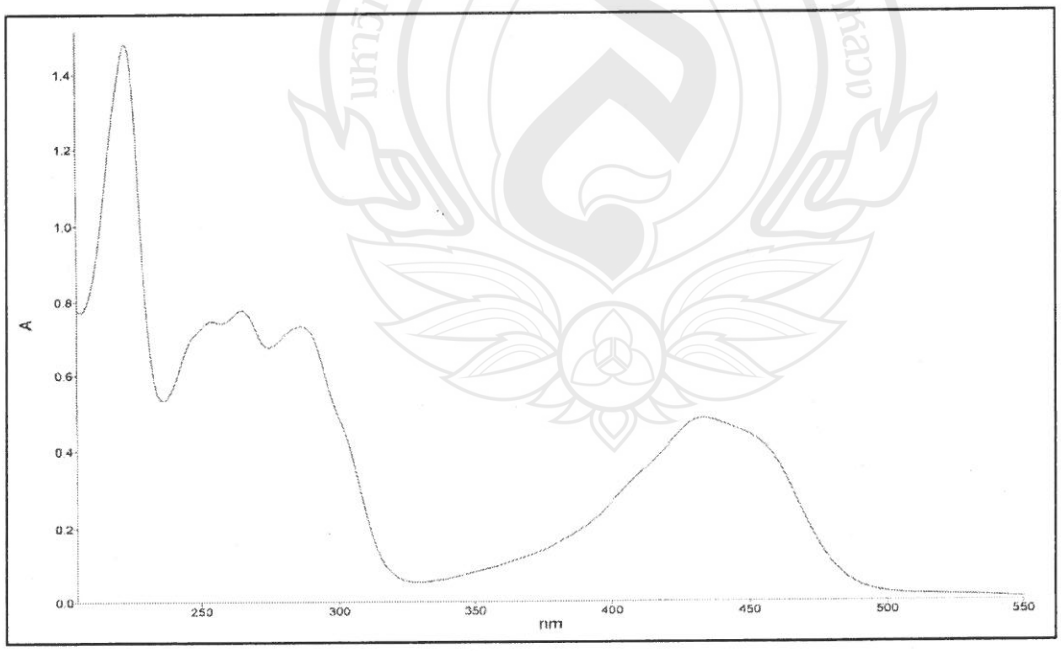
COSY (acetone-d_6) spectrum of **8**



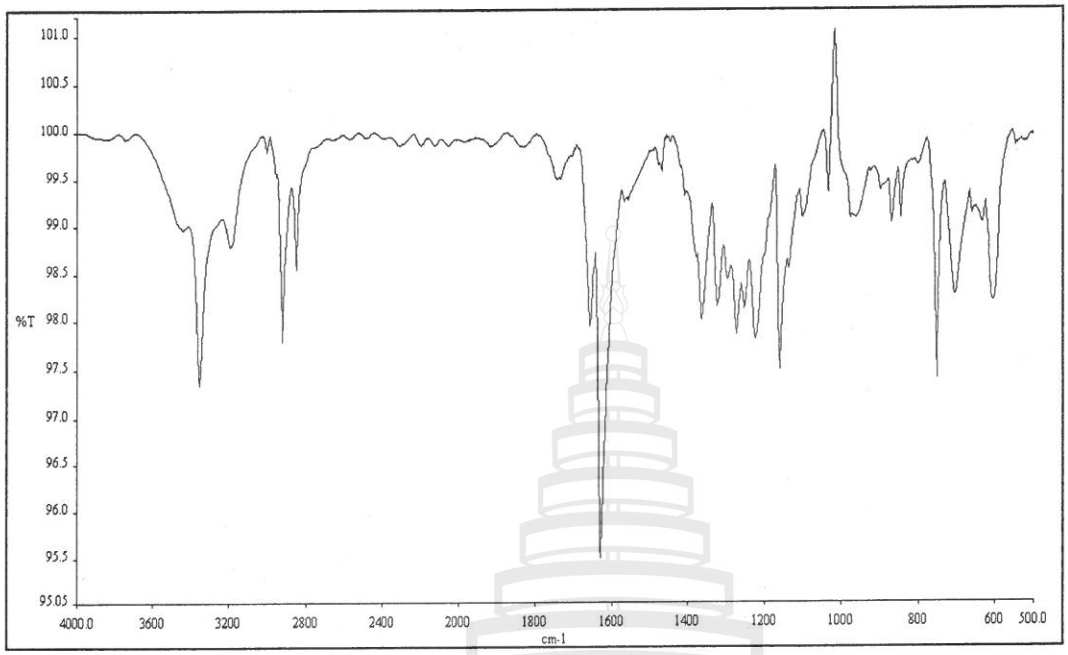
HMQC (acetone-d_6) spectrum of **8**



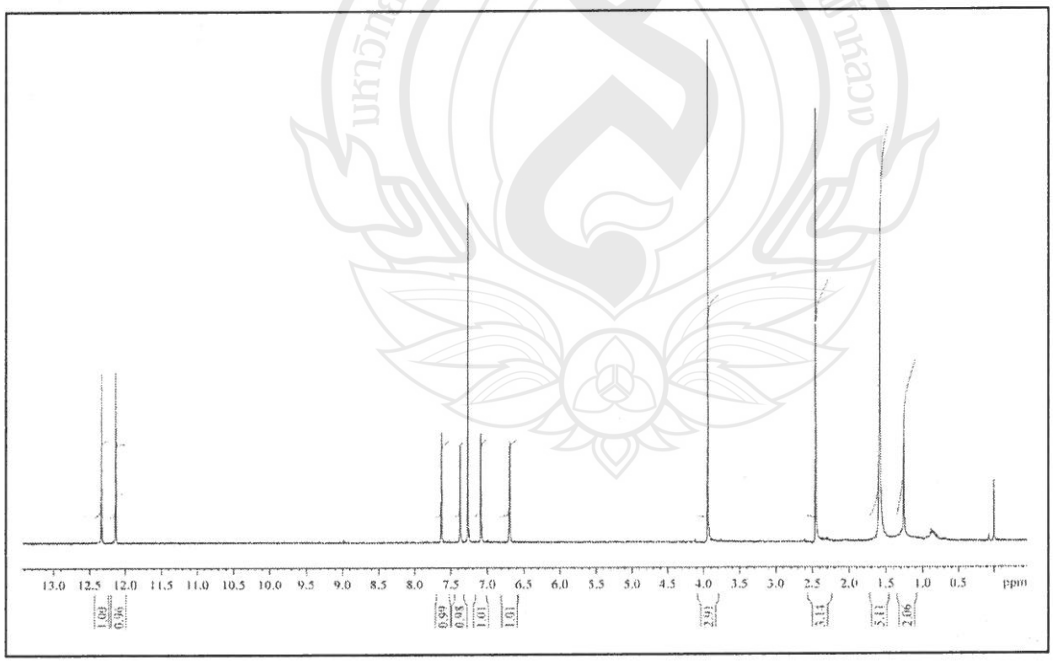
HMBC (acetone-d_6) spectrum of **8**



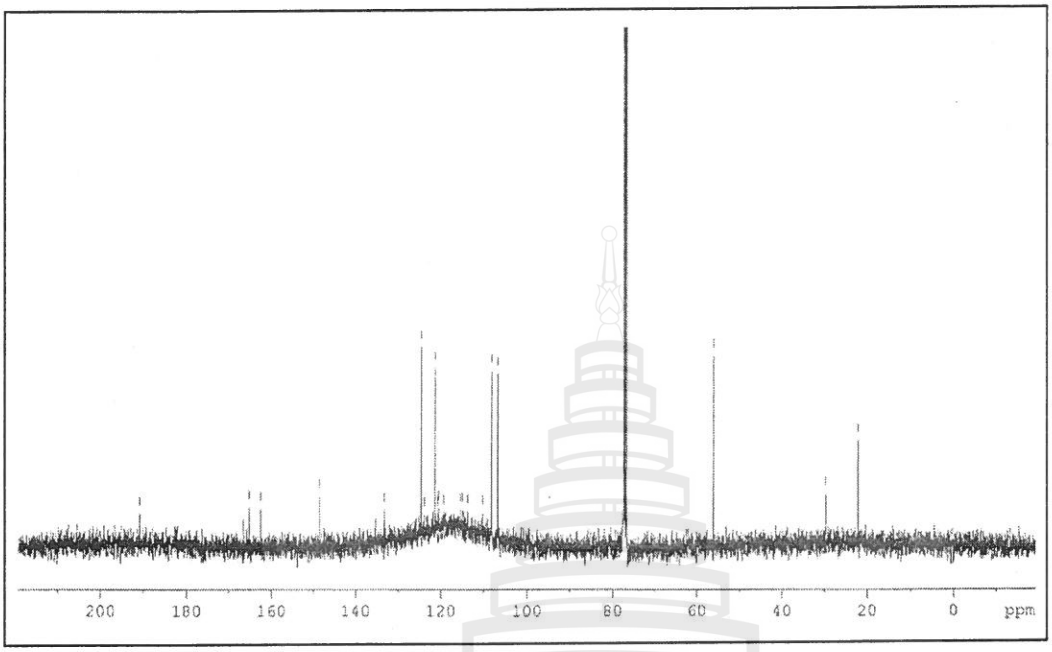
UV (MeOH) spectrum of **9**



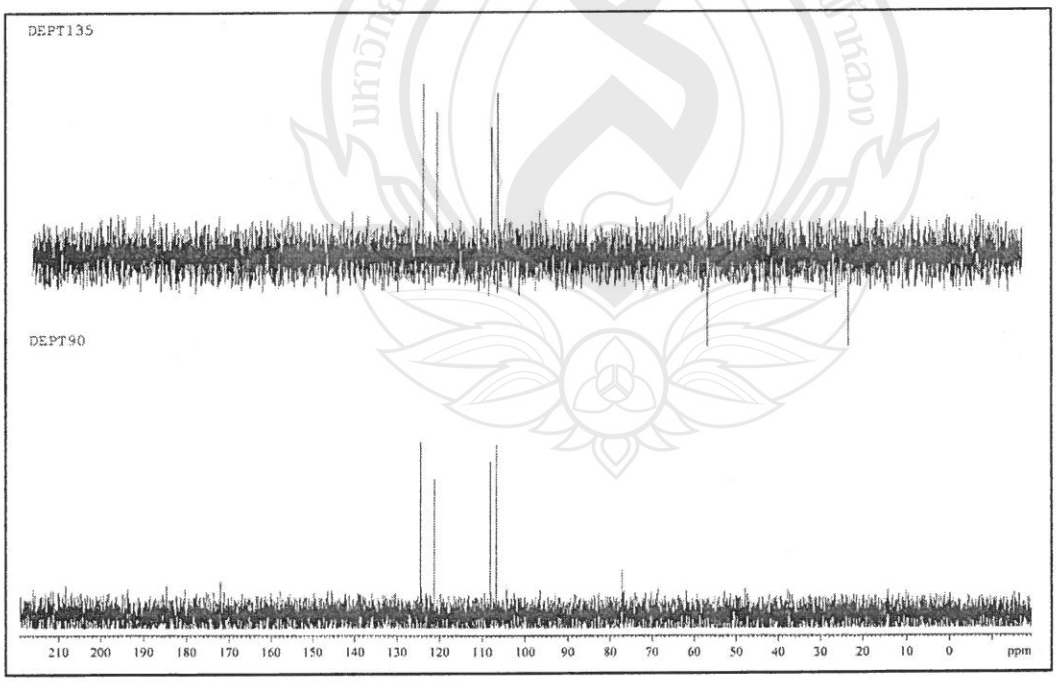
IR (KBr) spectrum of **9**



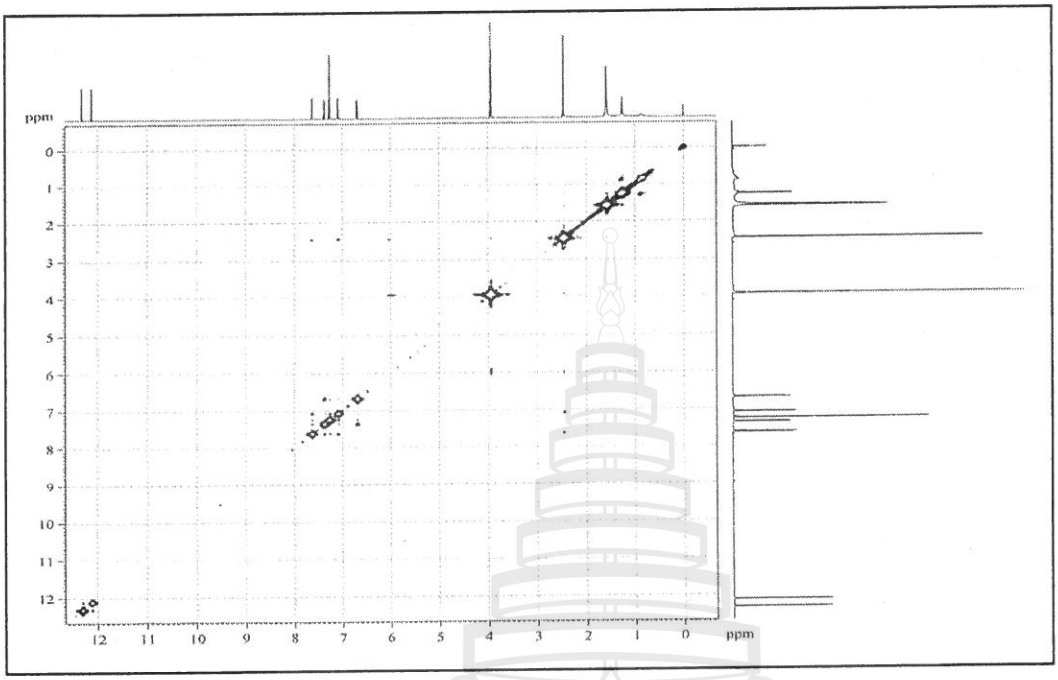
^{1}H NMR (400 MHz, acetone-d_6) spectrum of **9**



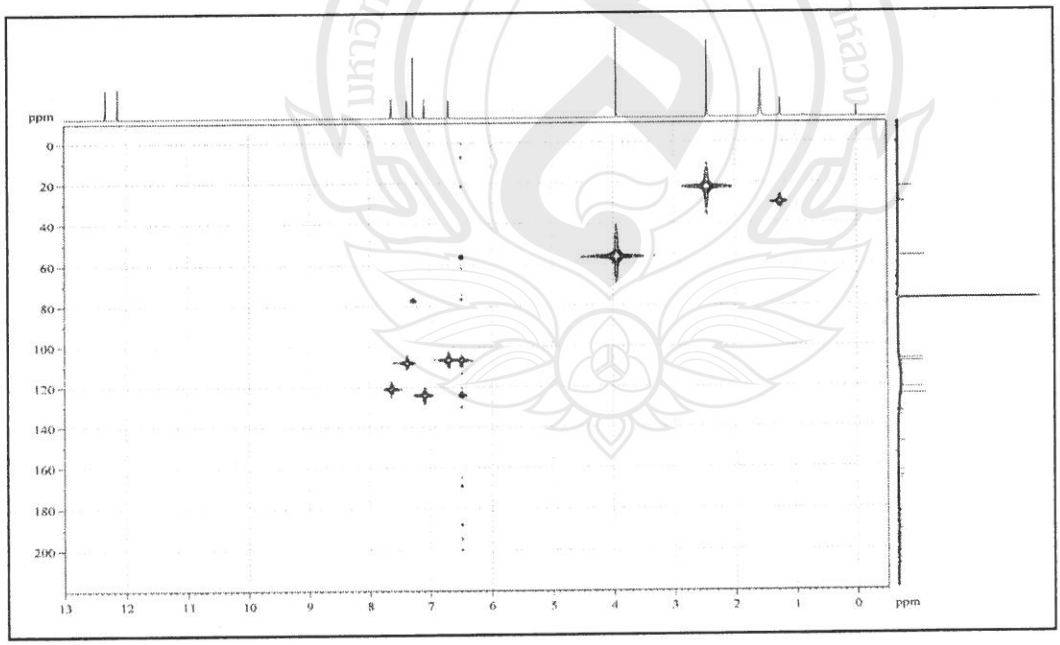
^{13}C NMR (100 MHz, acetone-d_6) spectrum of **9**



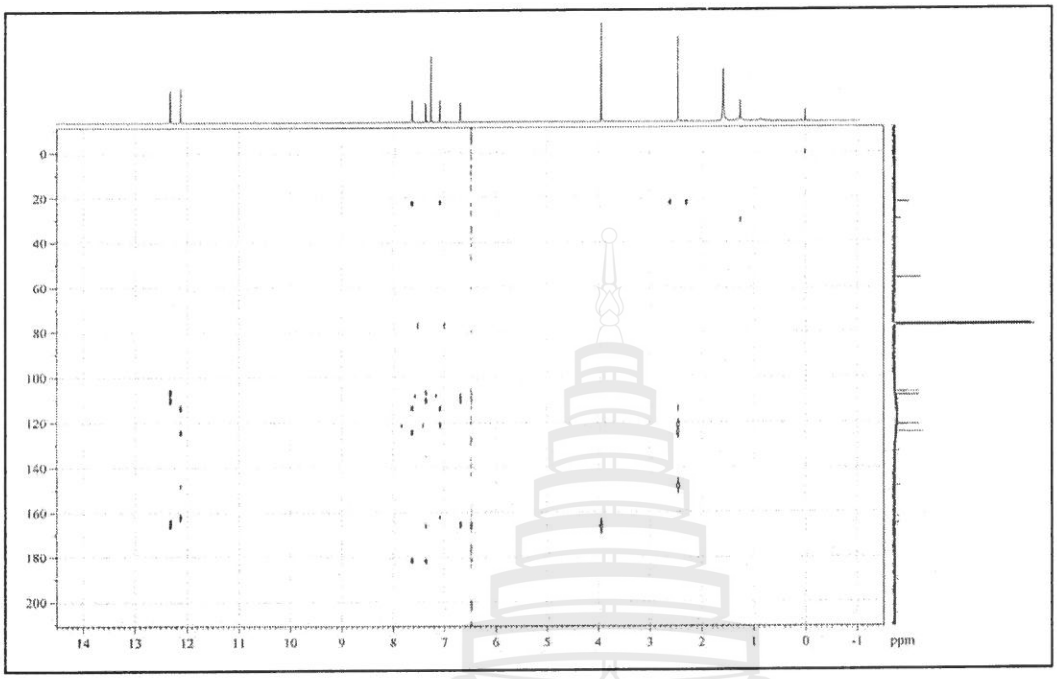
DEPT 135° and 90°(acetone-d_6) spectrum of **9**



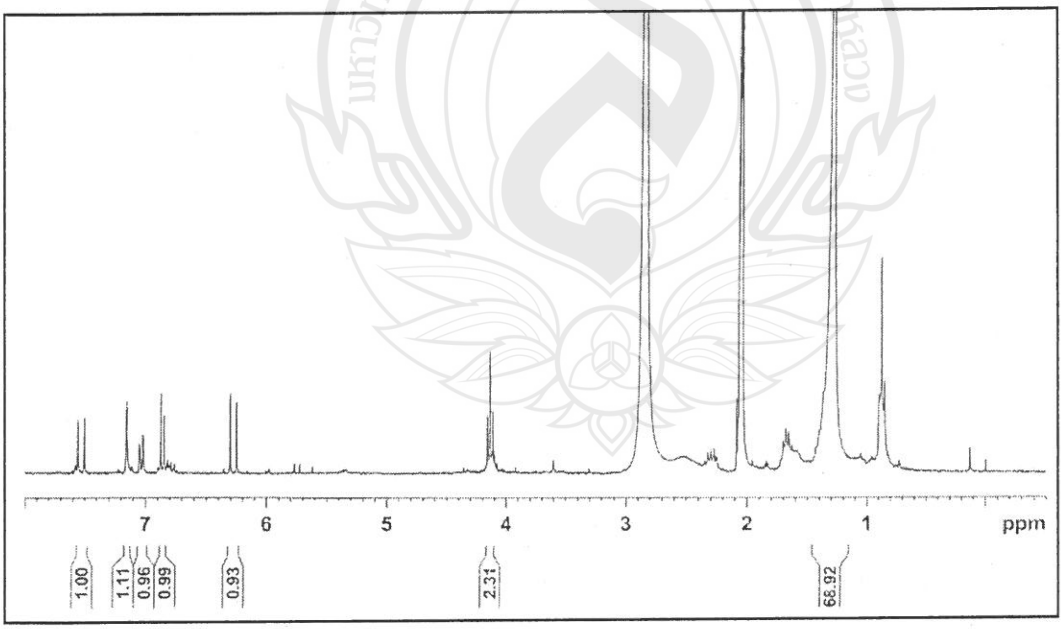
COSY (acetone-d_6)spectrum of **9**



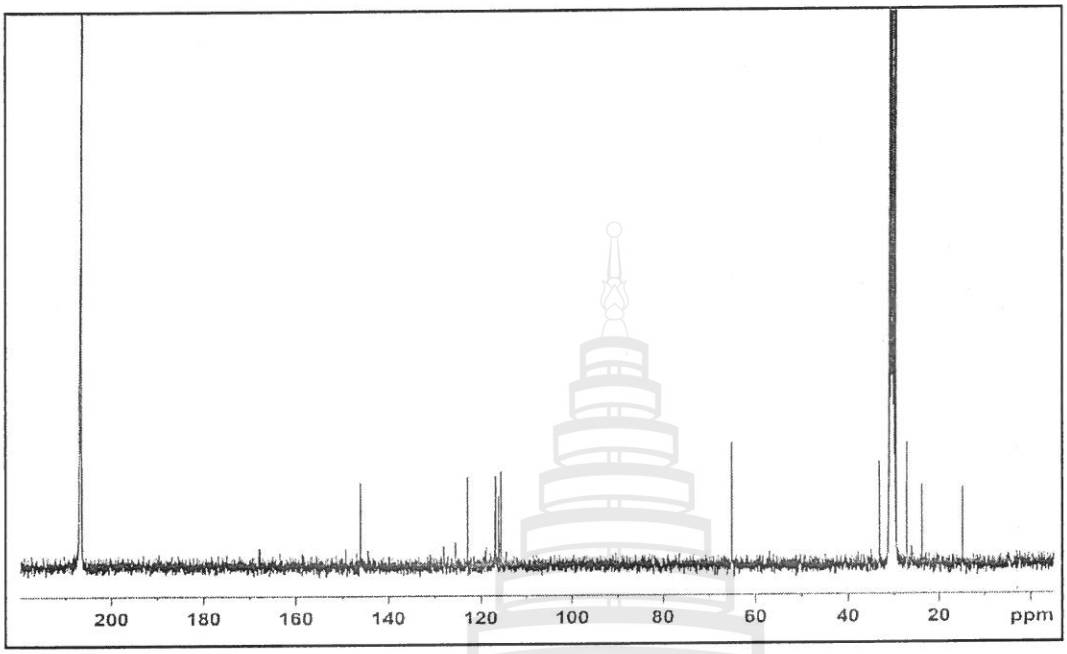
HMQC (acetone-d_6)spectrum of **9**



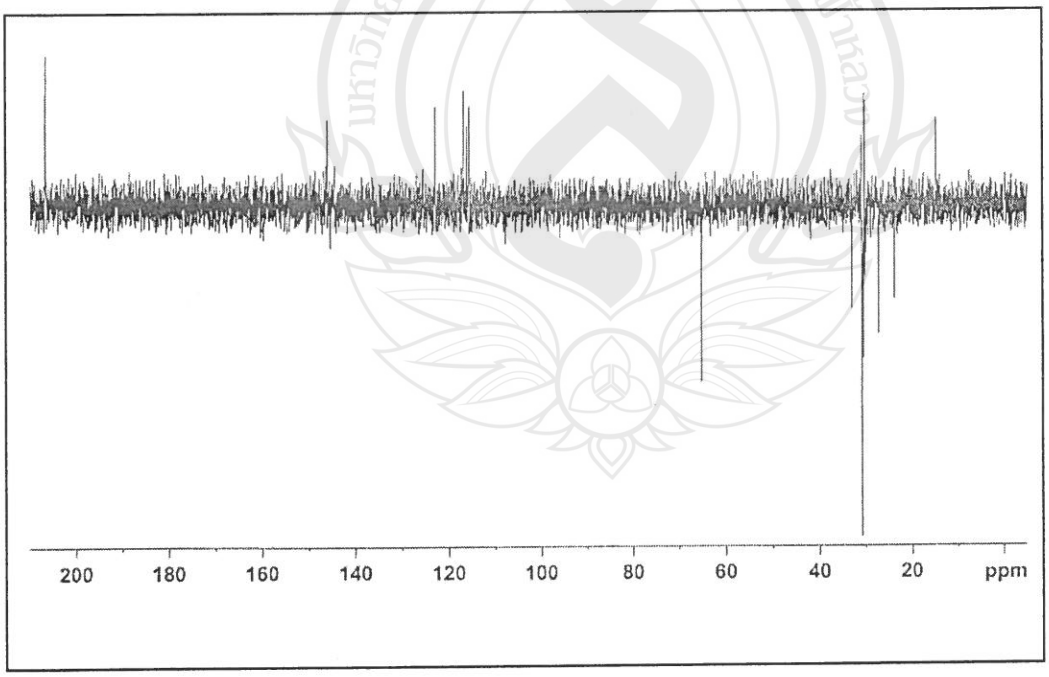
HMBC (acetone-d_6)spectrum of **9**



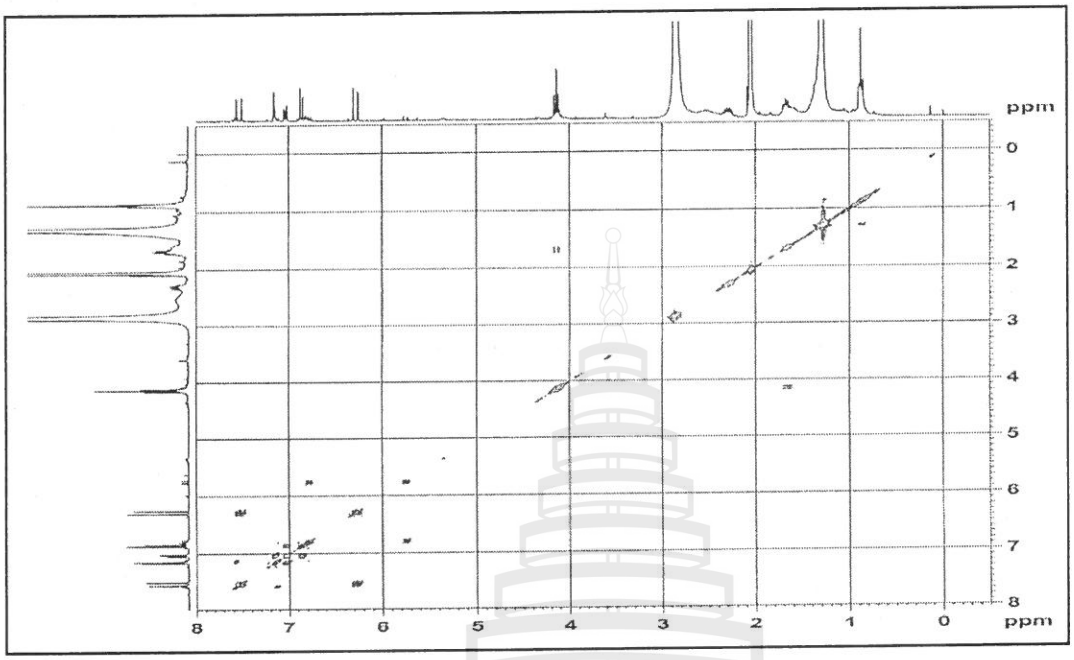
^{1}H NMR (300 MHz, acetone-d_6) spectrum of **12**



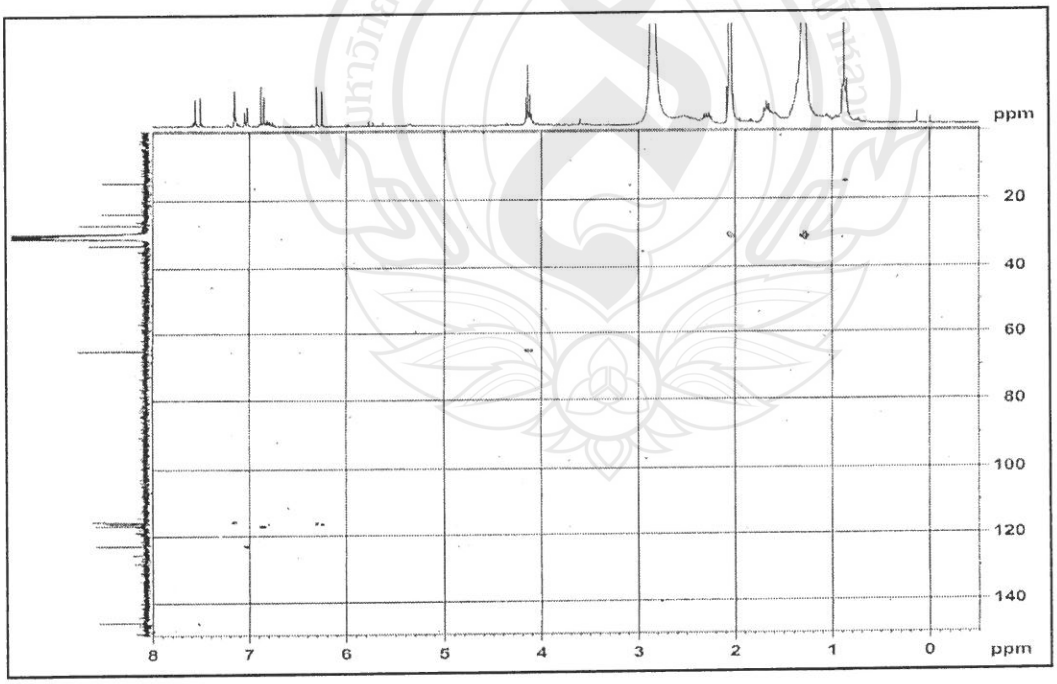
^{13}C NMR (75 MHz, acetone-d_6) spectrum of **12**



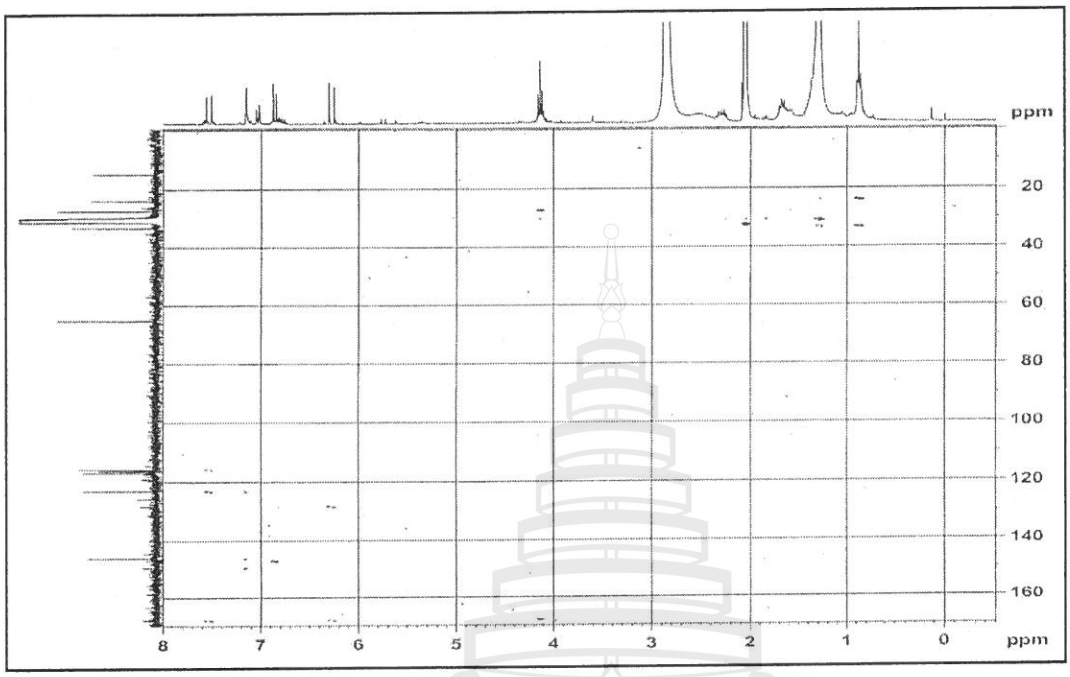
DEPT 135°(acetone-d_6) spectrum of **12**



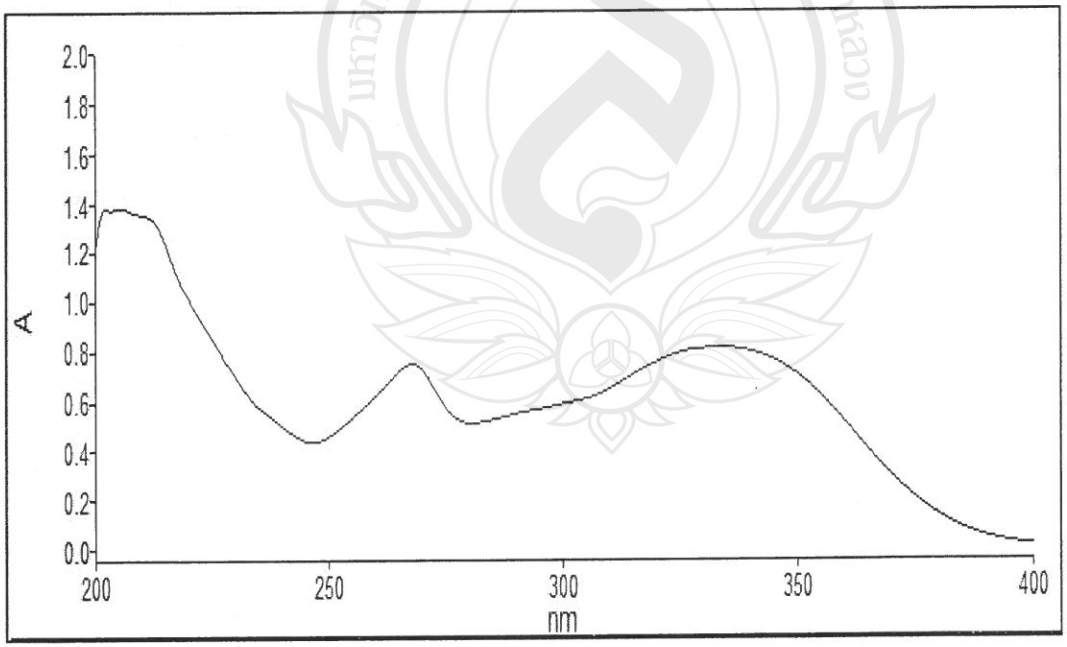
COSY (acetone-d_6) spectrum of **12**



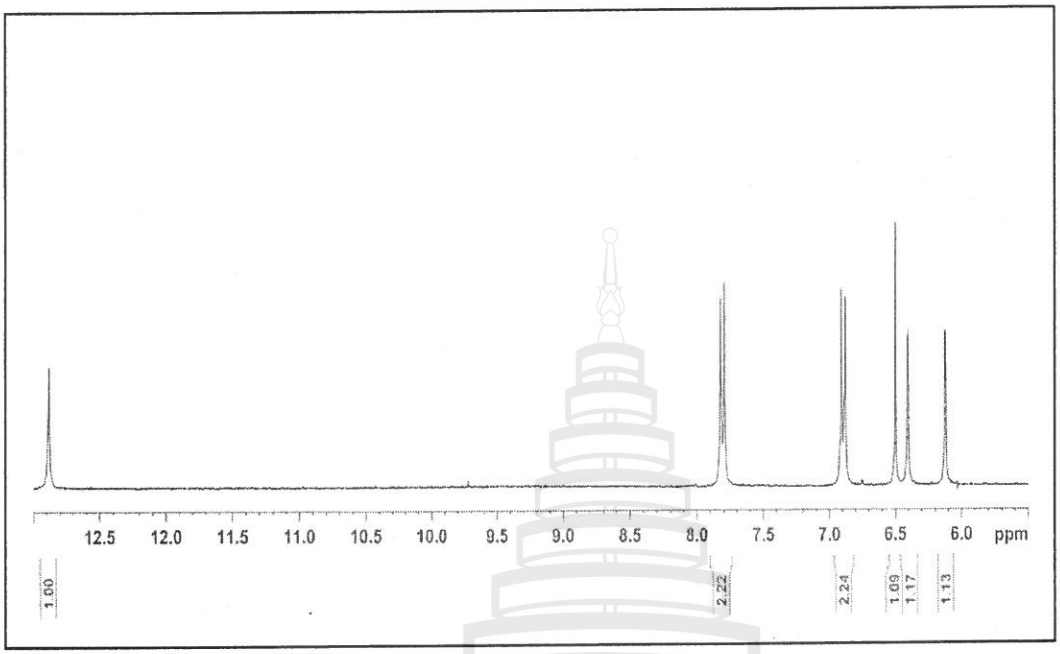
HMQC (acetone-d_6) spectrum of **12**



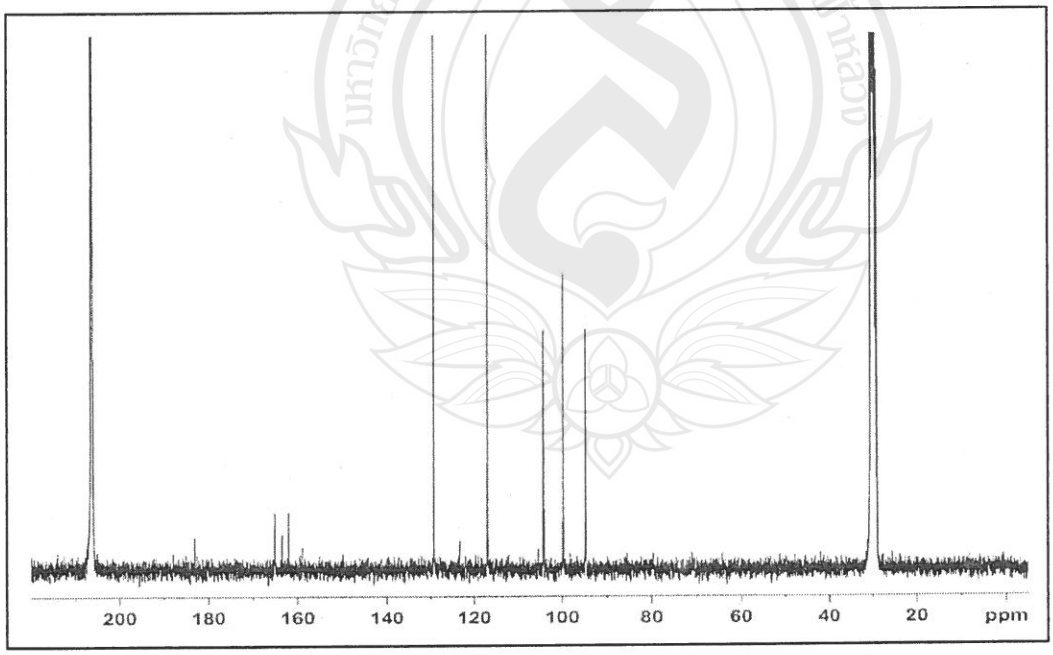
HMBC (acetone-d_6) spectrum of **12**



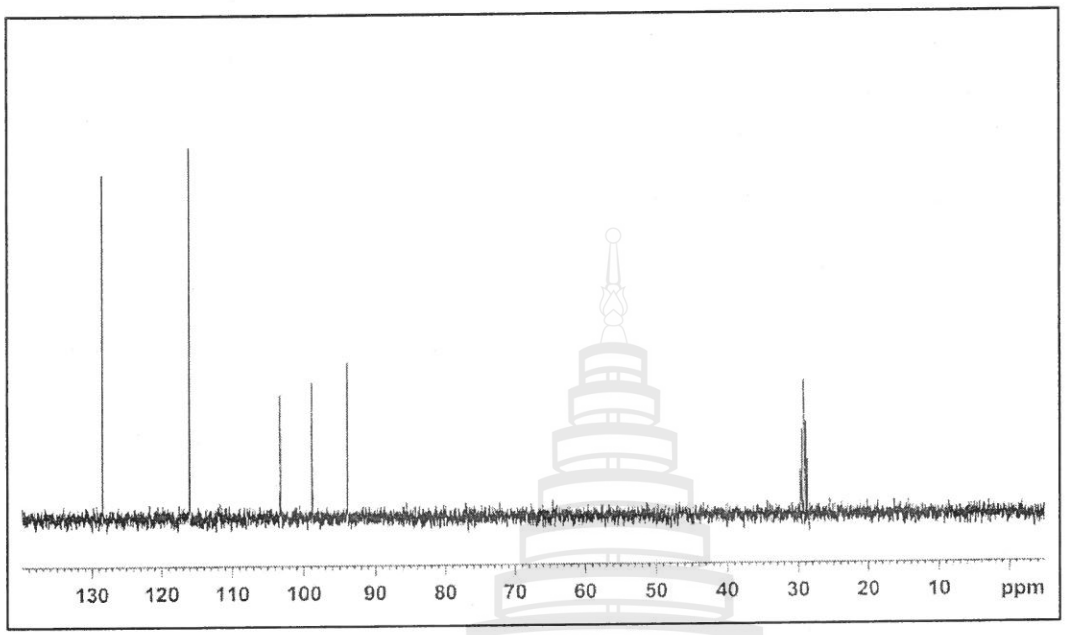
UV (MeOH) spectrum of **13**



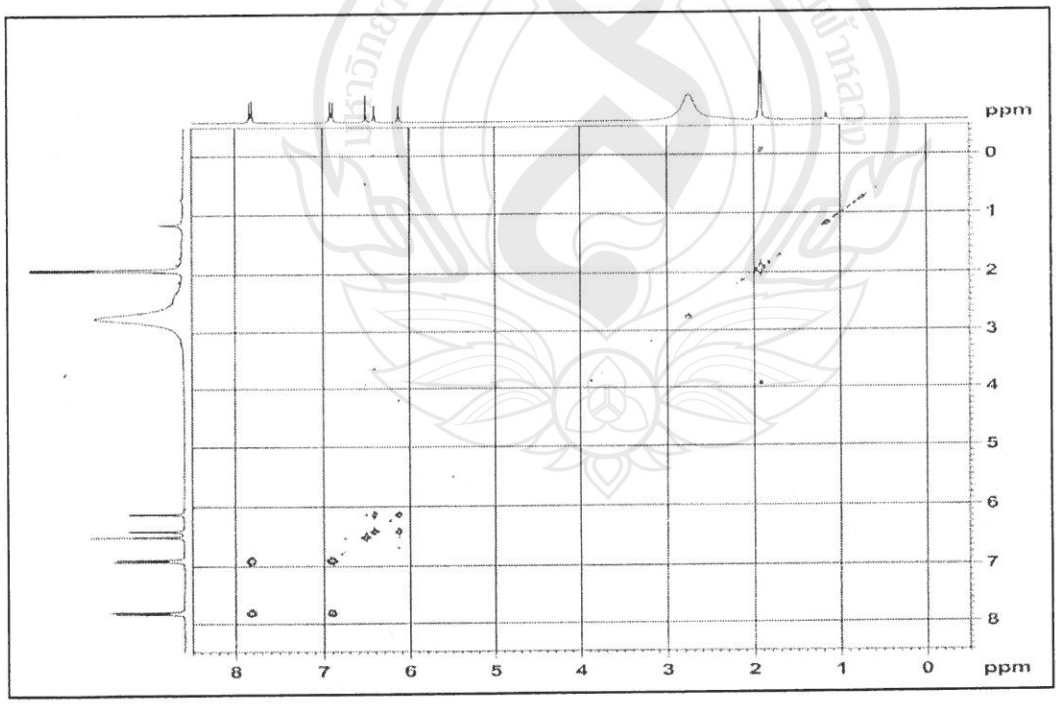
^{1}H NMR (300 MHz, acetone-d_6) spectrum of **13**



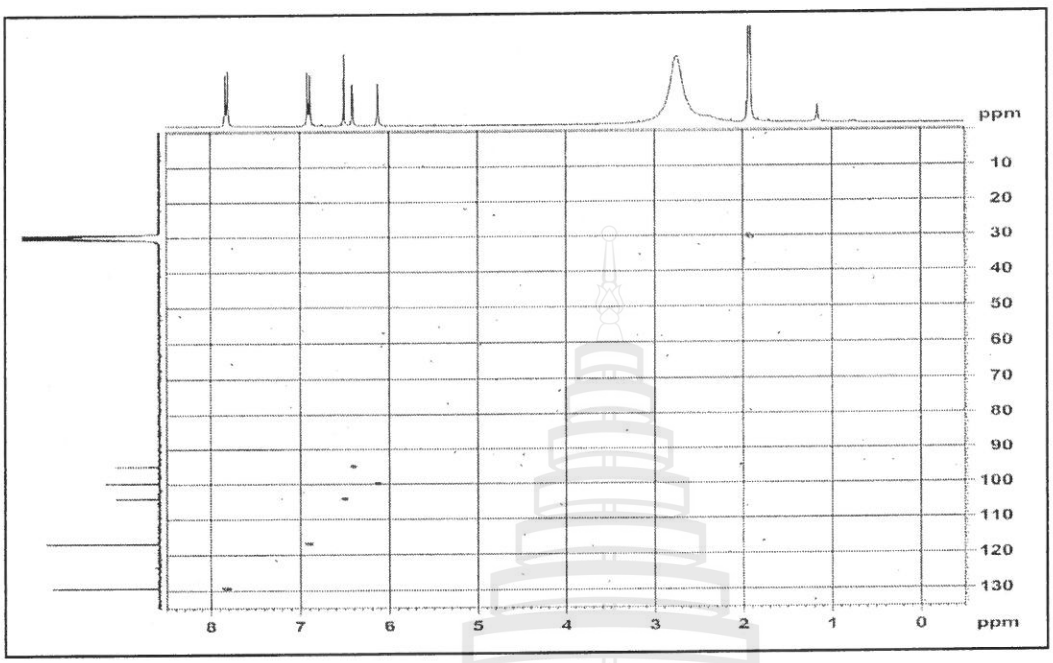
^{13}C NMR (75 MHz, acetone-d_6) spectrum of **13**



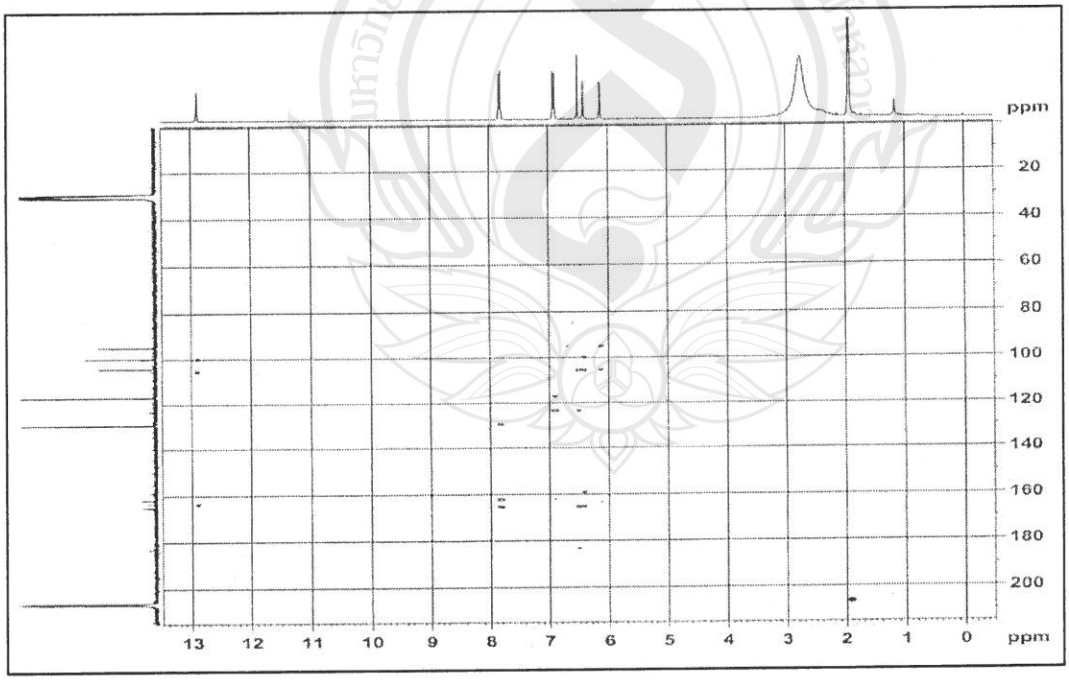
DEPT 135°(acetone-d_6) spectrum of **13**



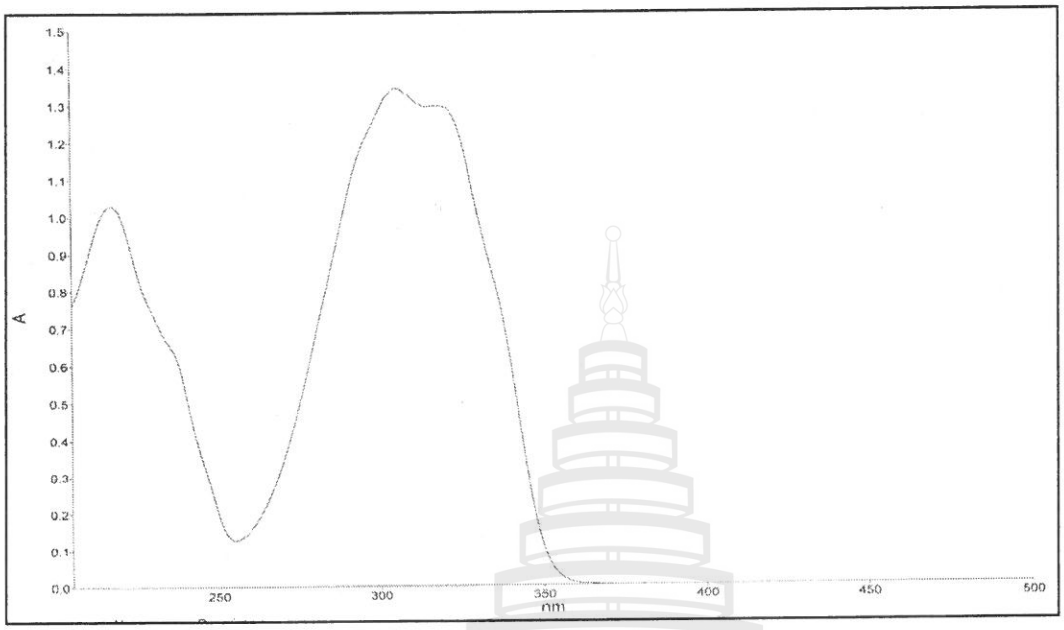
COSY (acetone-d_6) spectrum of **13**



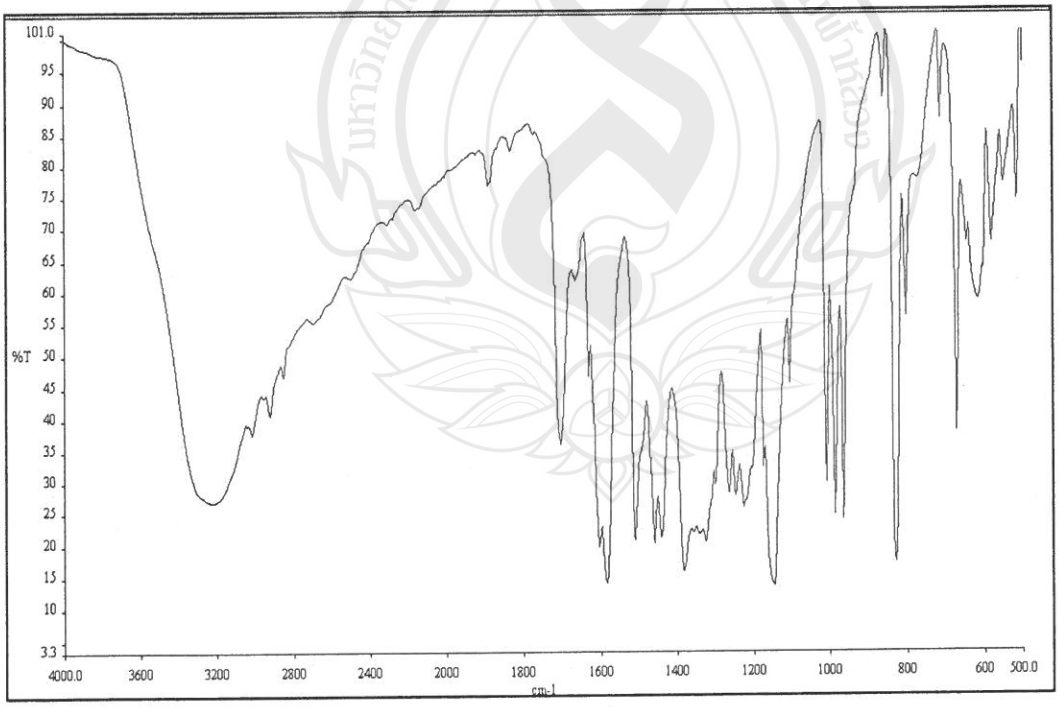
HMQC (acetone-d_6) spectrum of **13**



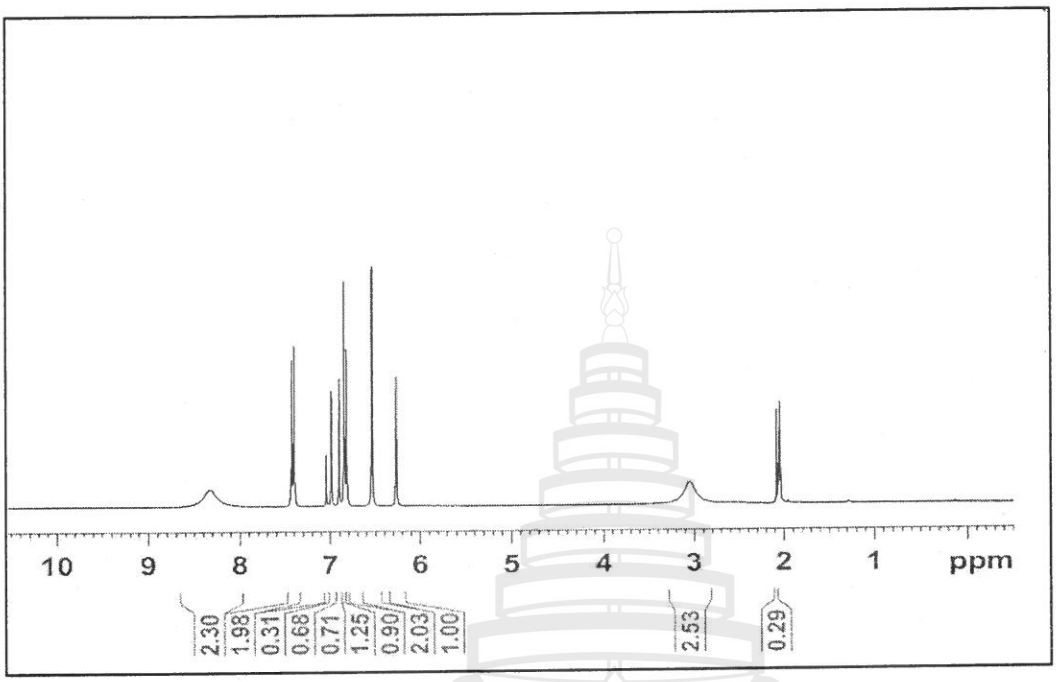
HMBC (acetone-d_6) spectrum of **13**



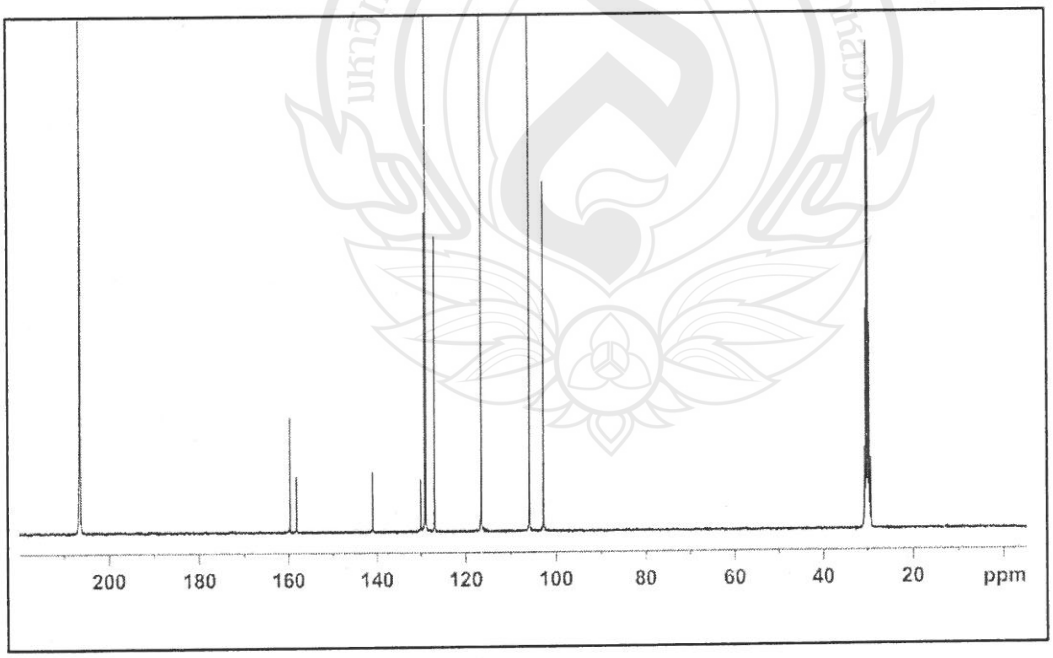
UV (MeOH) spectrum of **14**



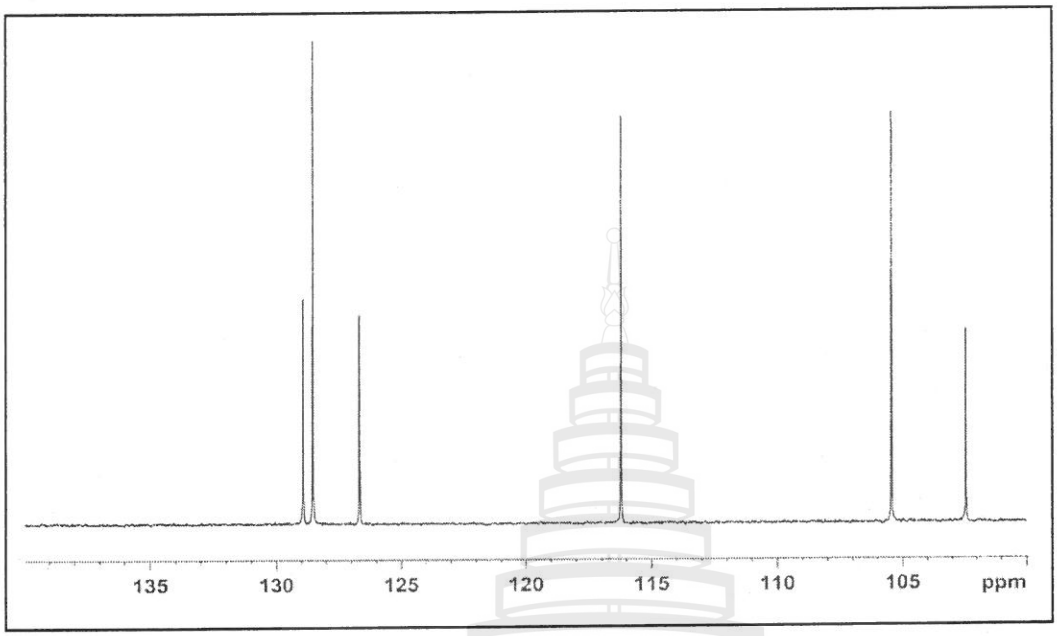
IR (KBr) spectrum of **14**



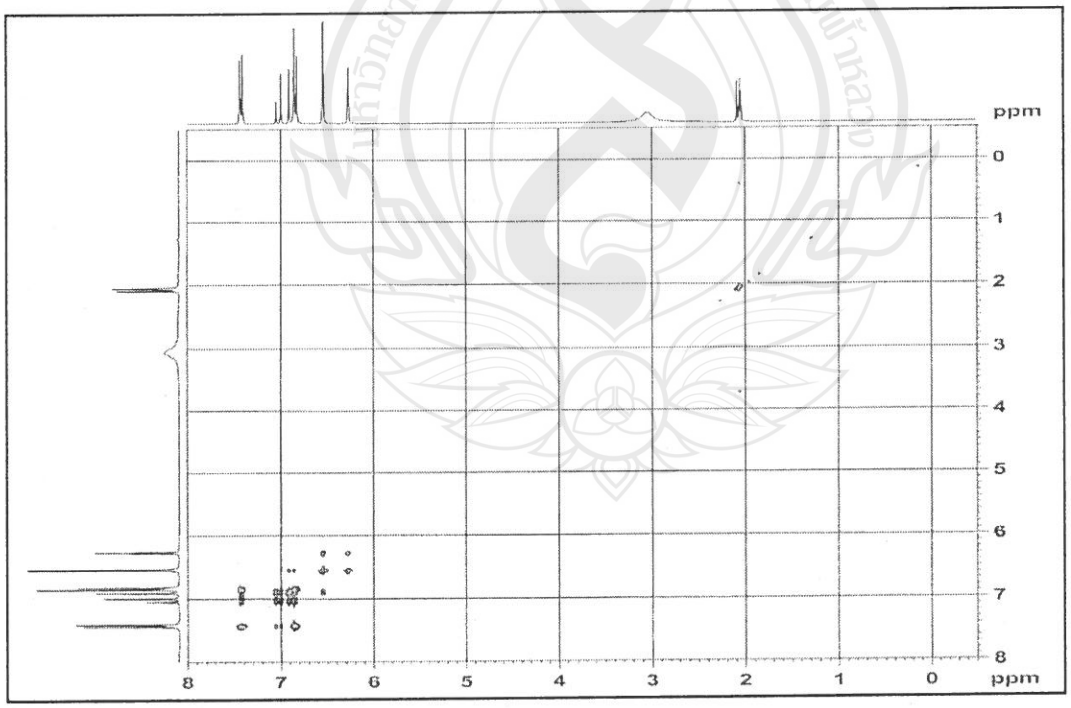
^{1}H NMR (300 MHz, acetone-d_6) spectrum of **14**



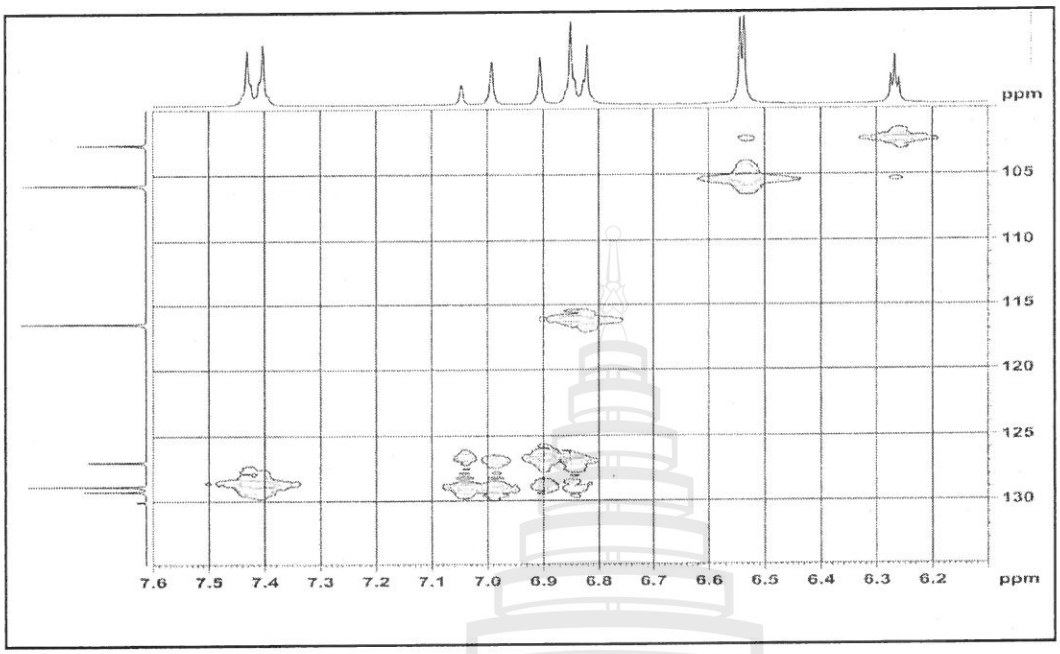
^{13}C NMR (75 MHz, acetone-d_6) spectrum of **14**



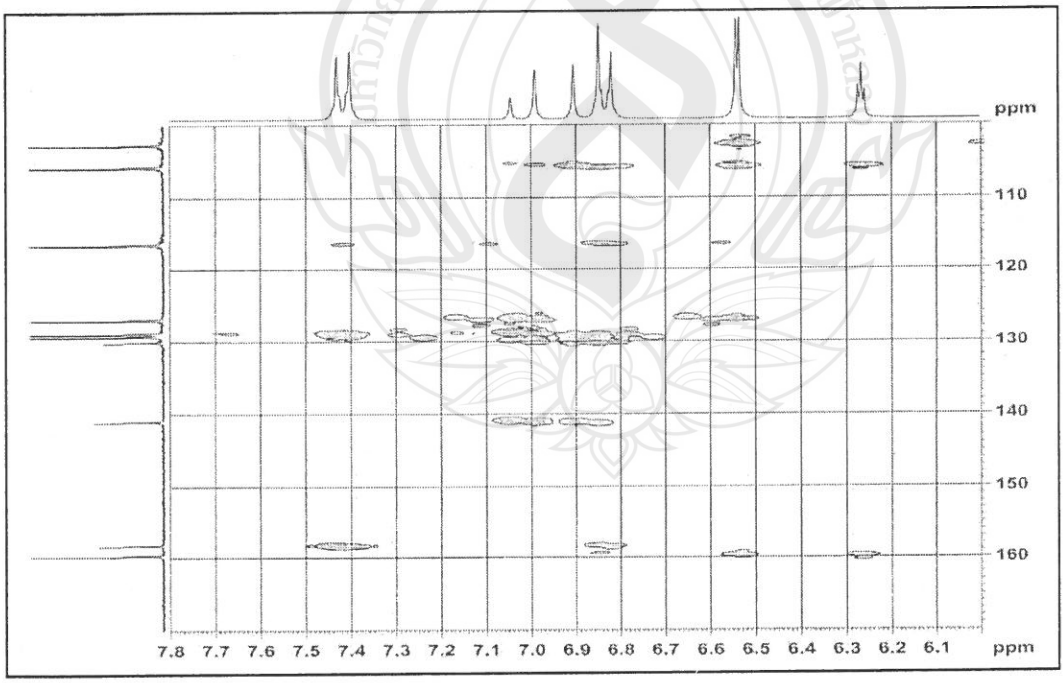
DEPT 90°(acetone-d_6)spectrum of **14**



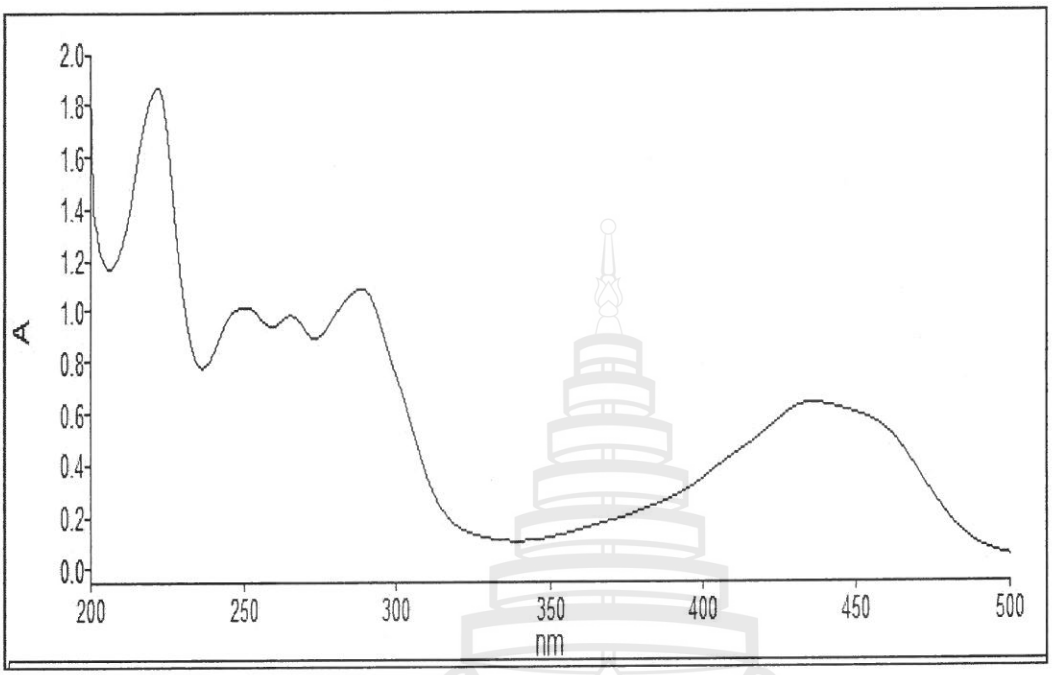
COSY (acetone-d_6) spectrum of **14**



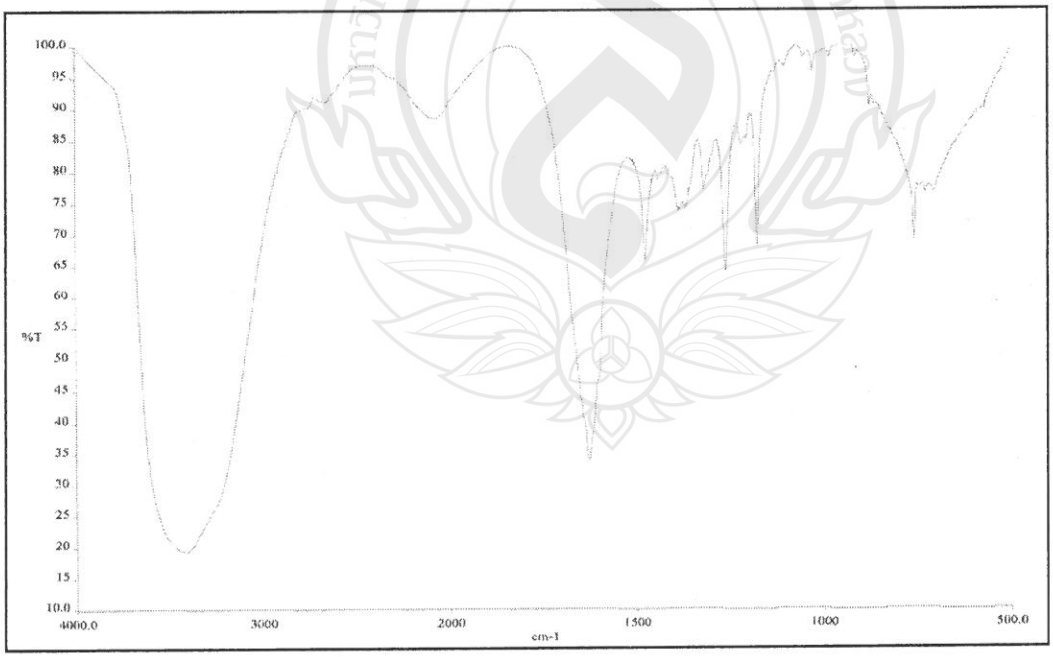
HMQC (acetone-d_6) spectrum of **14**



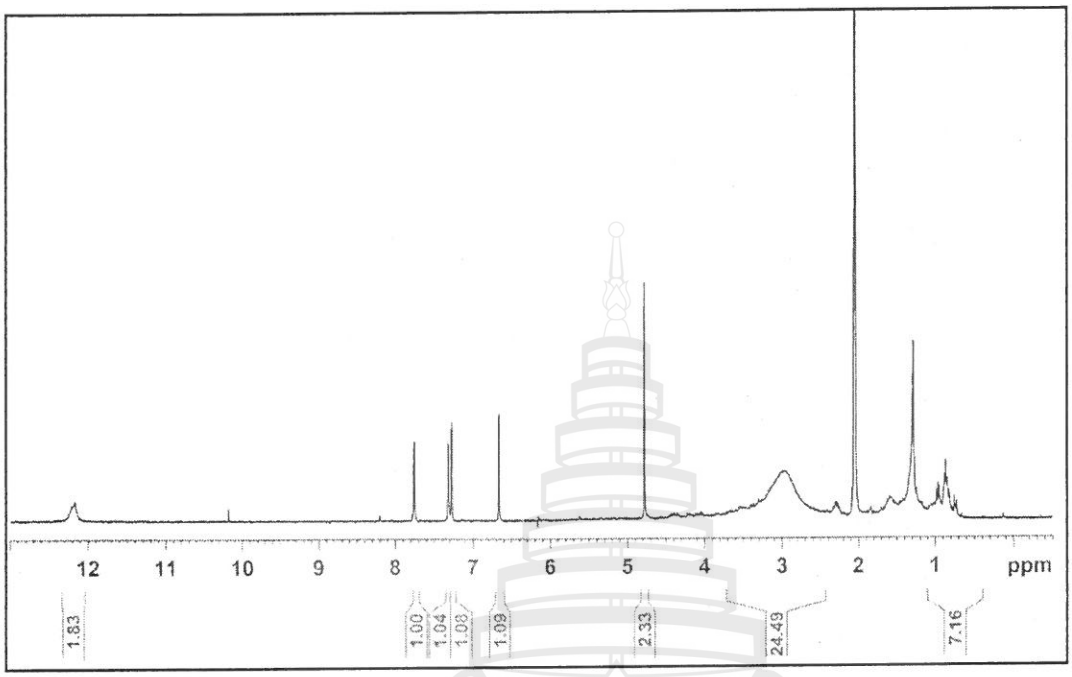
HMBC (acetone-d_6) spectrum of **14**



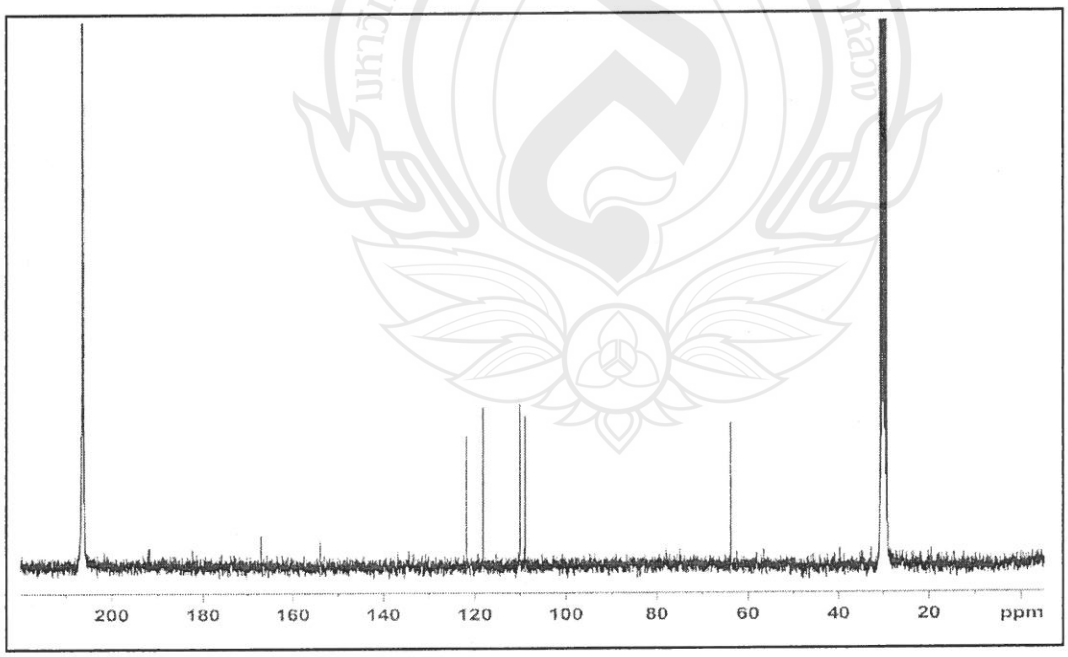
UV (MeOH) spectrum of **15**



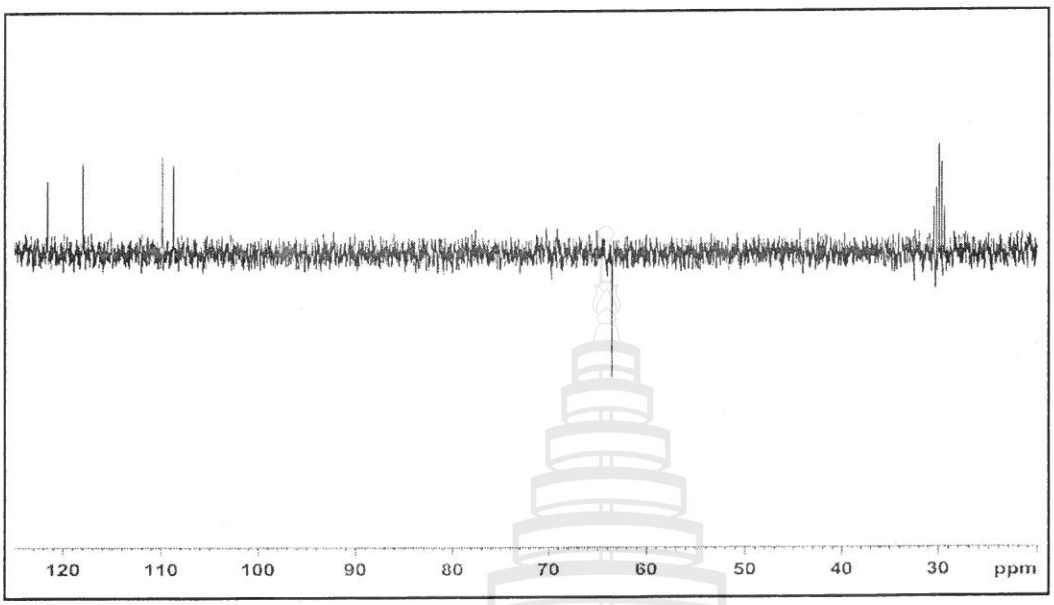
IR (KBr) spectrum of **15**



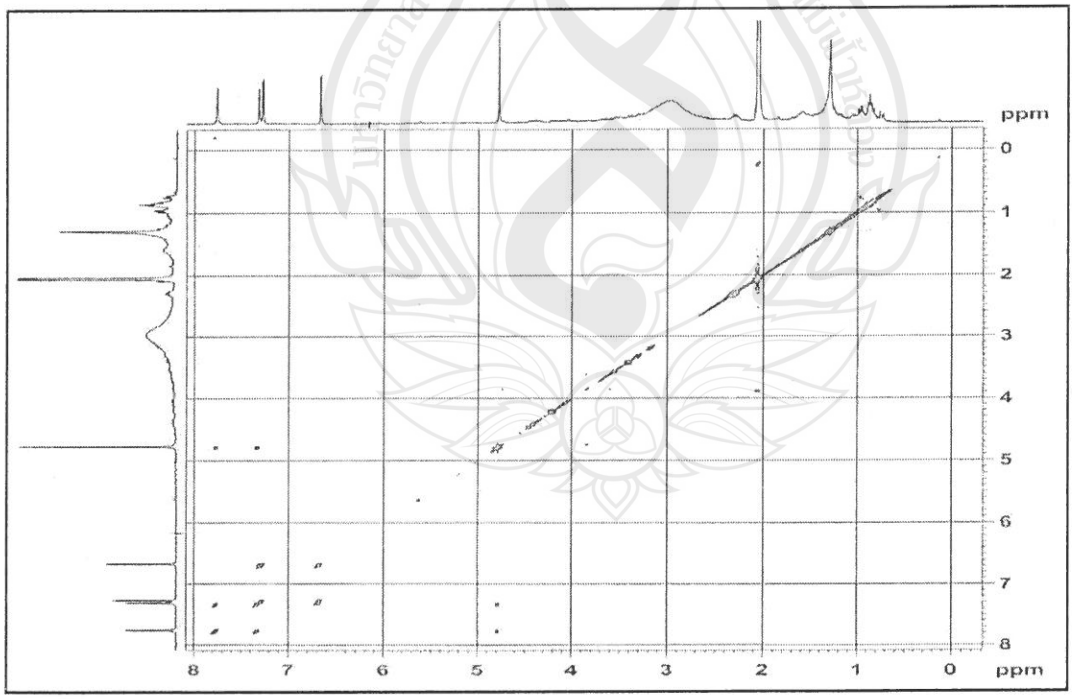
^{1}H NMR (300 MHz, acetone-d_6) spectrum of **15**



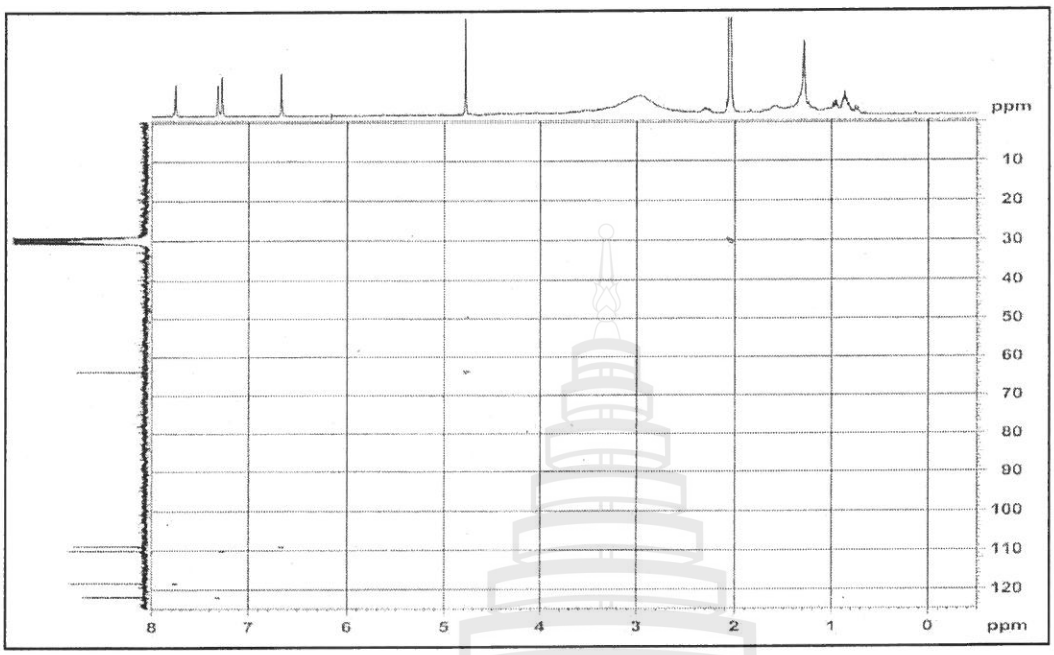
^{13}C NMR (75 MHz, acetone-d_6) spectrum of **15**



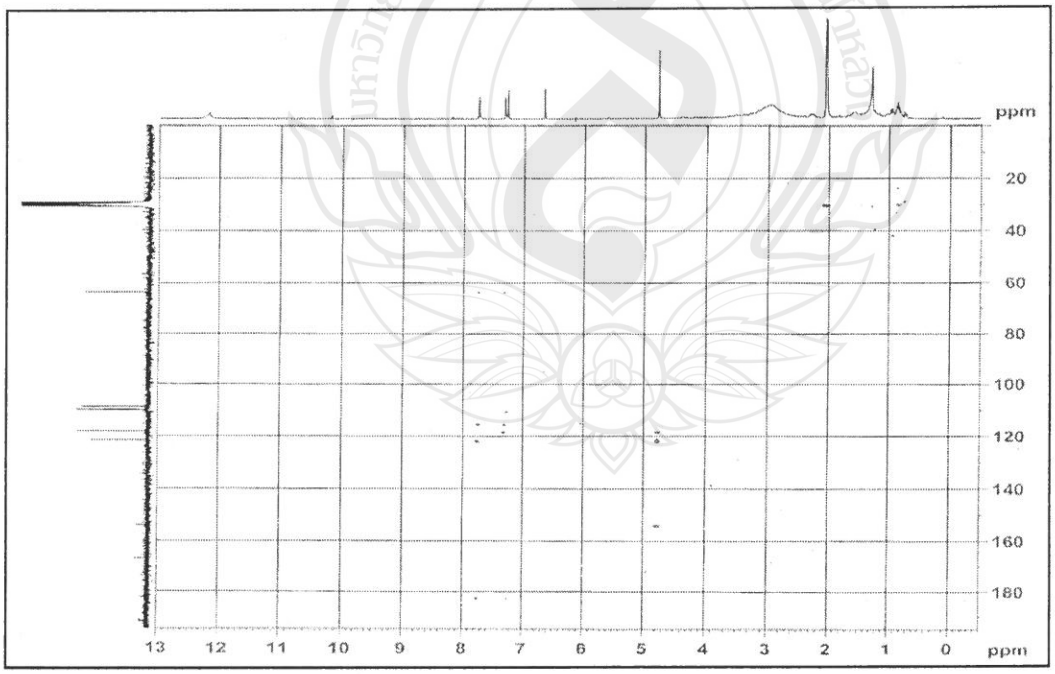
DEPT 135°(acetone-d_6) spectrum of **15**



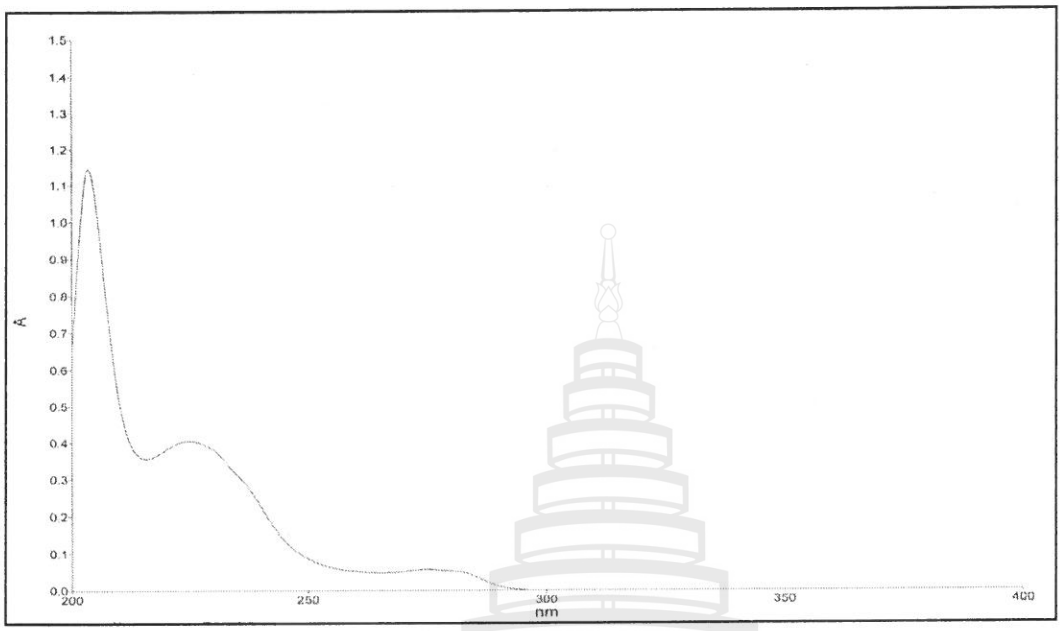
COSY (acetone-d_6) spectrum of **15**



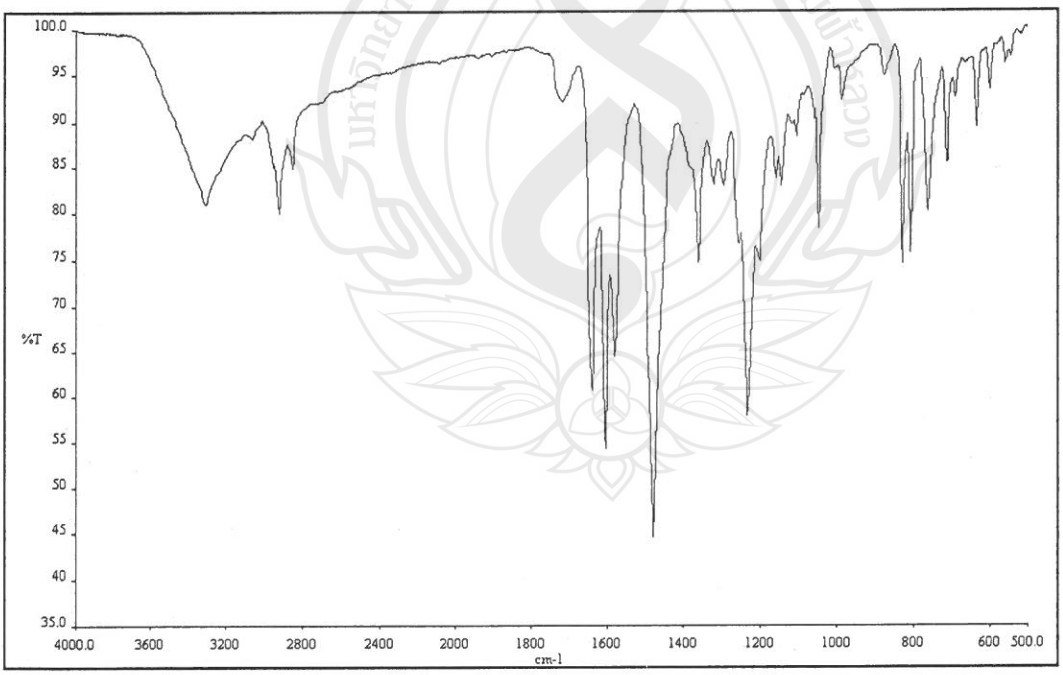
HMQC (acetone-d_6) spectrum of **15**



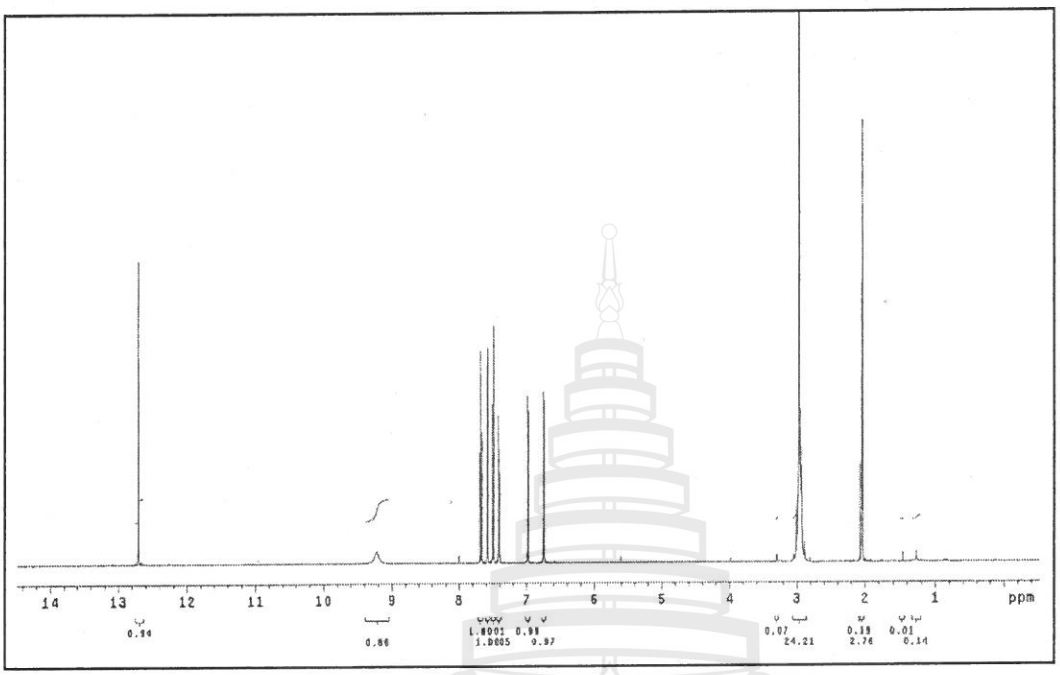
HMBC (acetone-d_6) spectrum of **15**



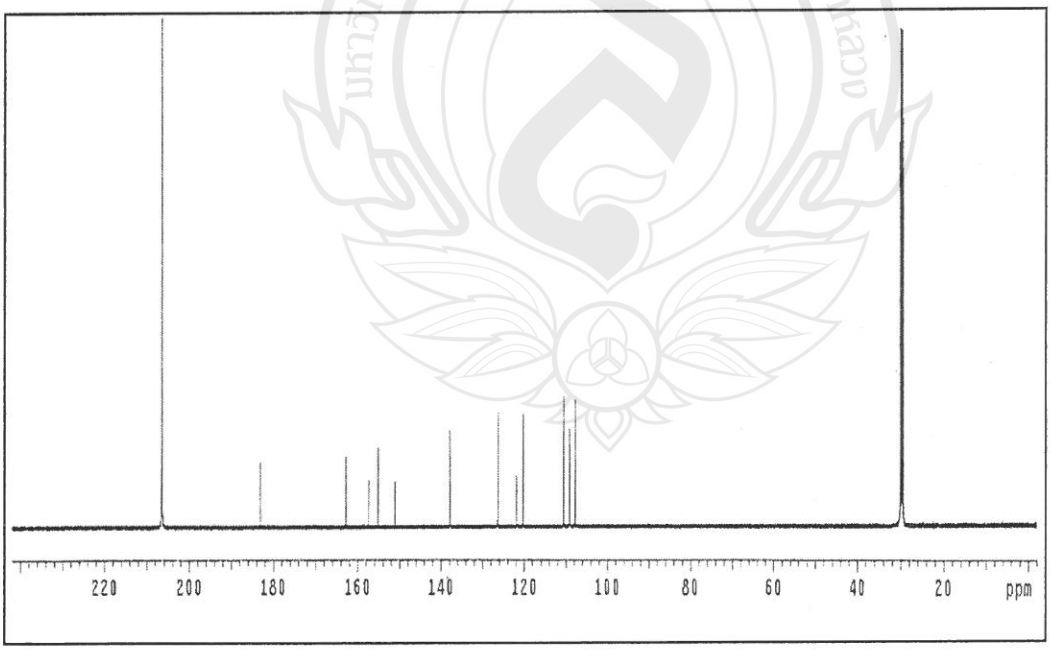
UV (MeOH) spectrum of **17**



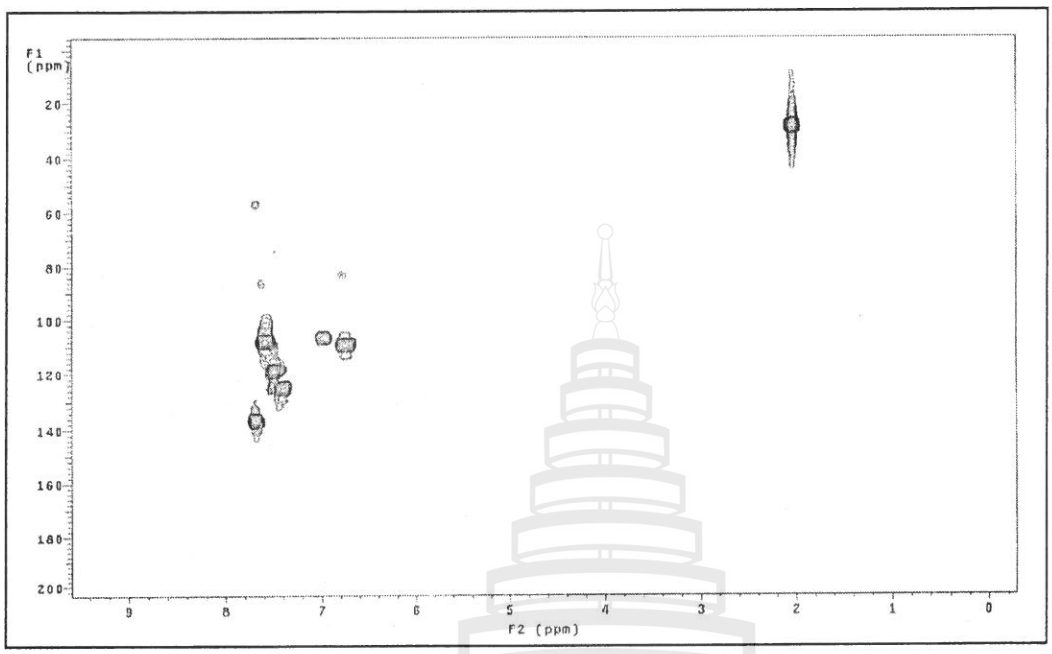
IR (KBr) spectrum of **17**



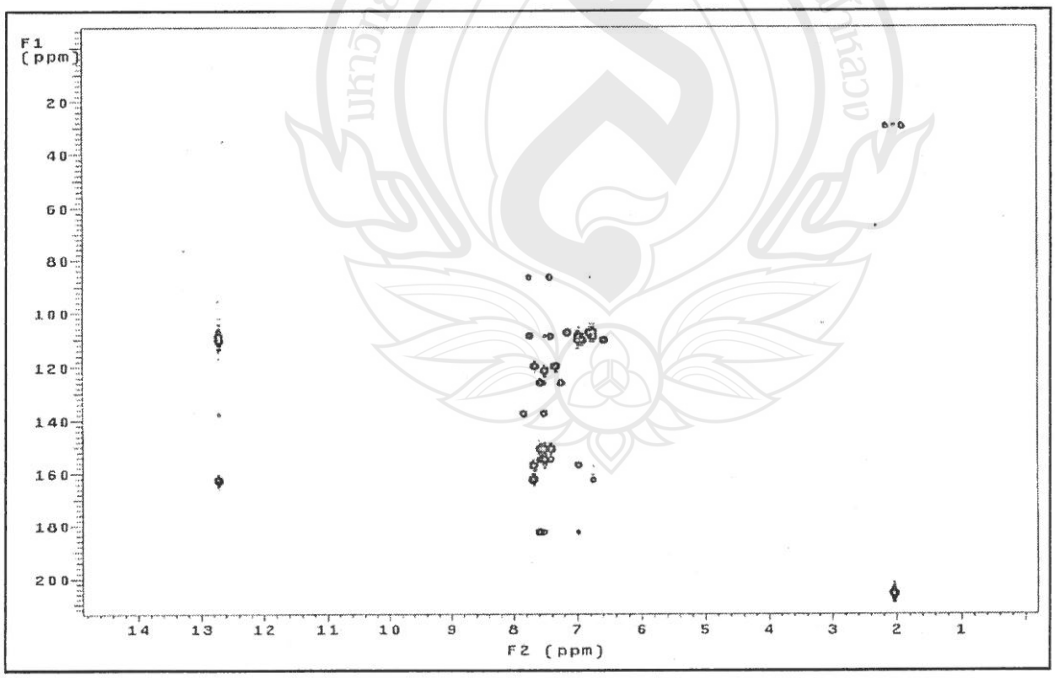
^{1}H NMR (400 MHz, acetone-d_6)spectrum of **17**



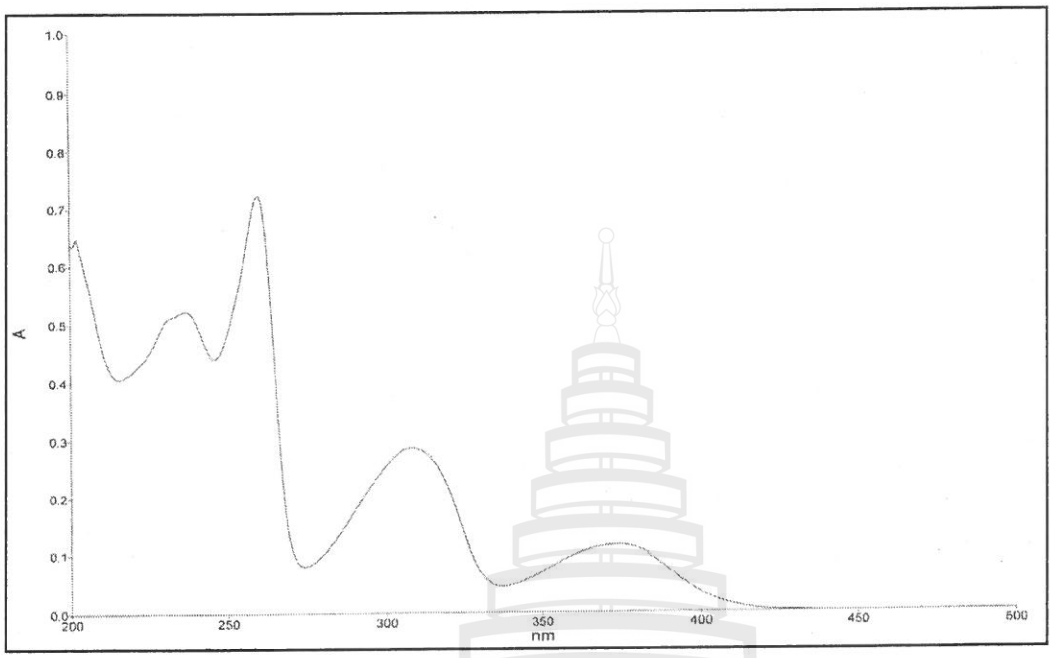
^{13}C NMR (100 MHz, acetone-d_6) spectrum of **17**



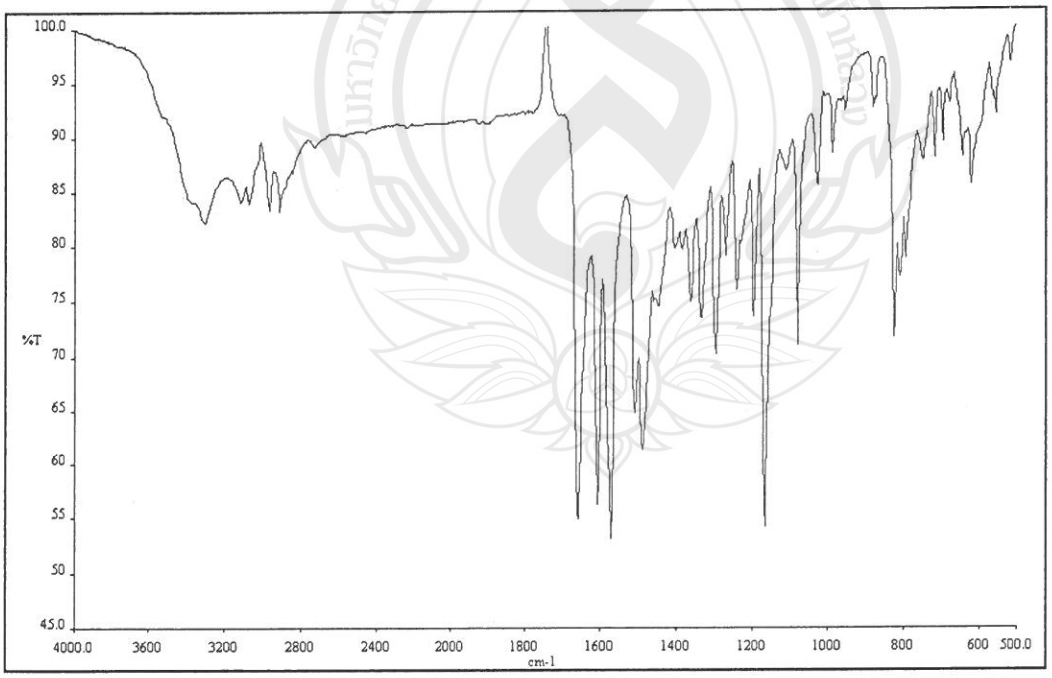
HMQC (acetone-d_6) spectrum of **17**



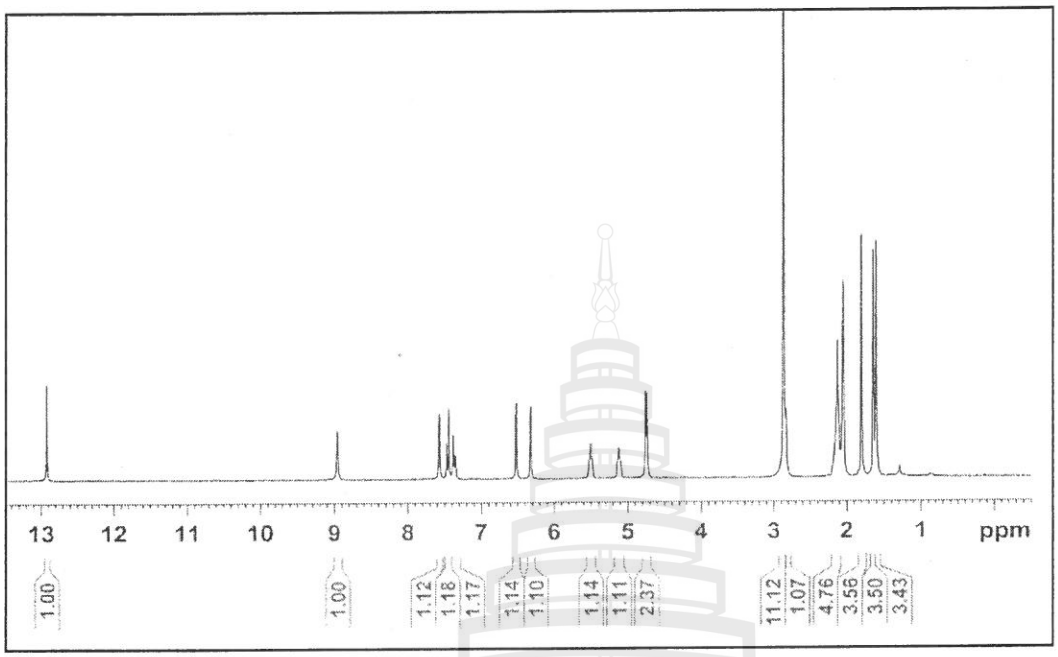
HMBC (acetone-d_6) spectrum of **17**



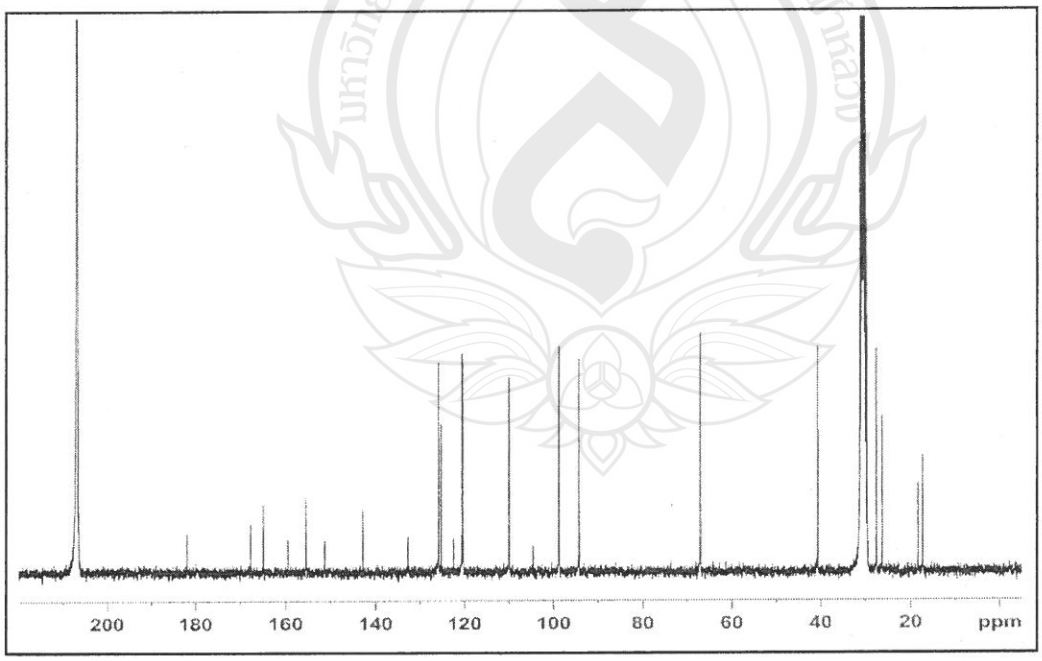
UV (MeOH) spectrum of **18**



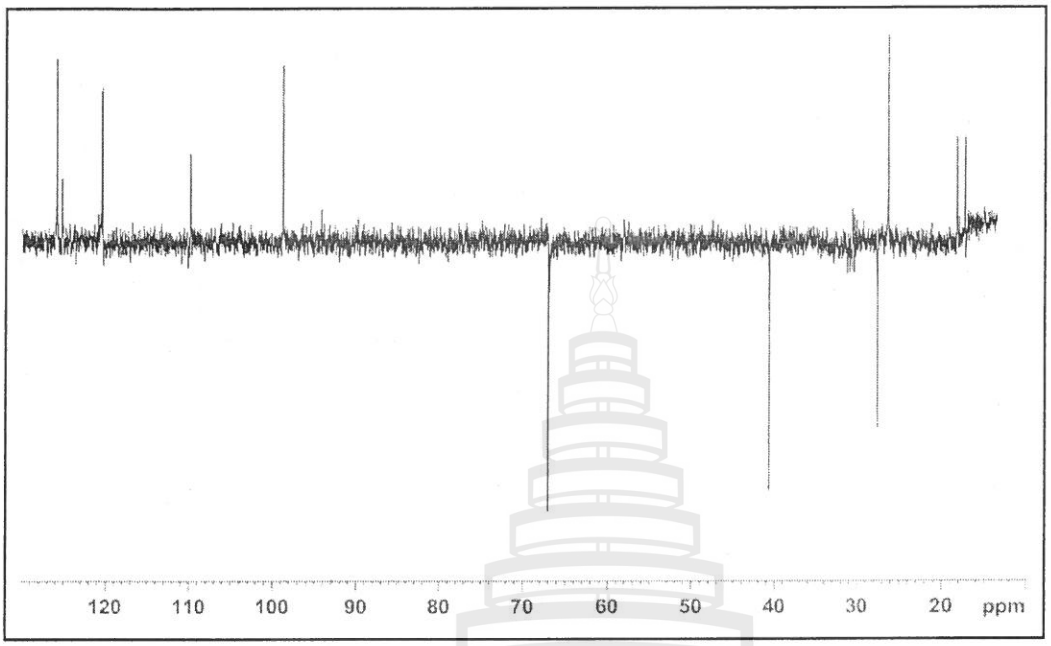
IR (KBr) spectrum of **18**



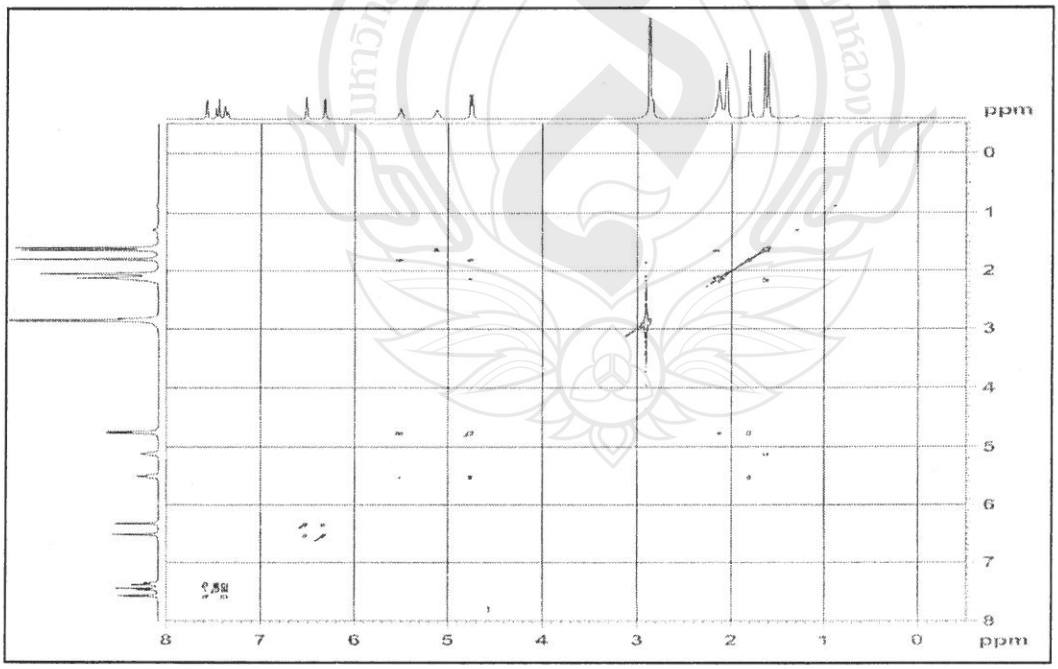
^{1}H NMR (300 MHz, acetone-d_6) spectrum of **18**



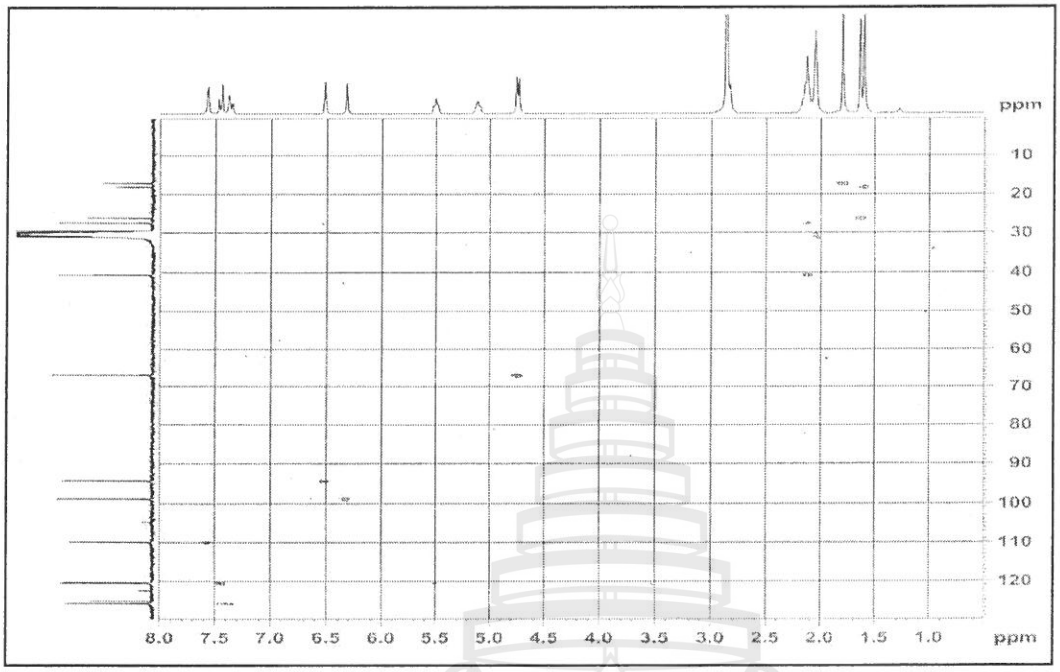
^{13}C NMR (75 MHz, acetone-d_6) spectrum of **18**



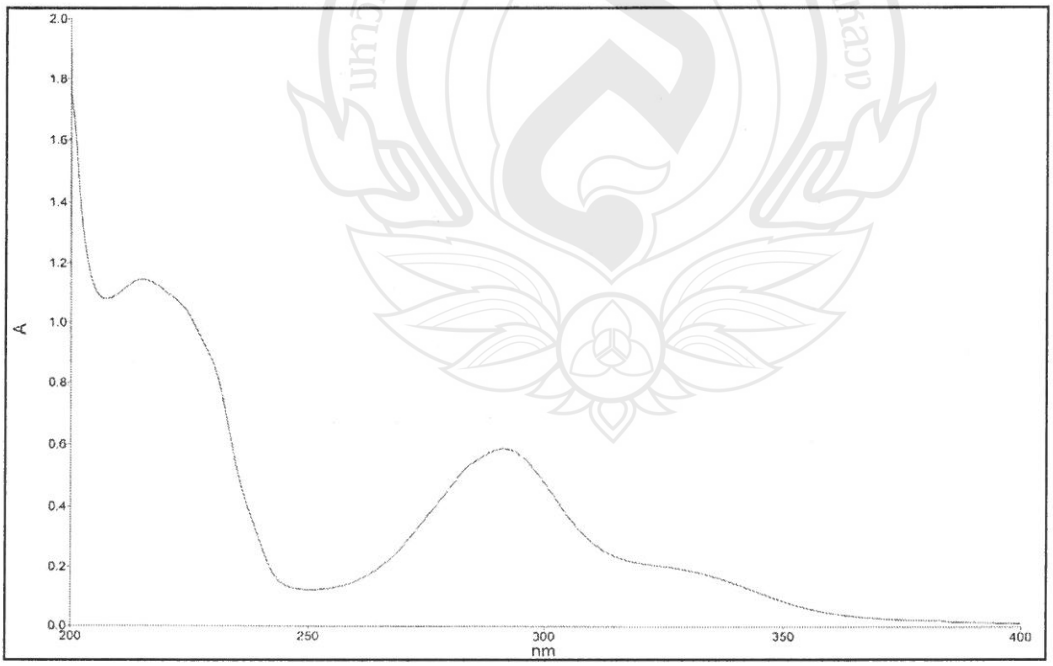
DEPT 135°(acetone-d_6) spectrum of **18**



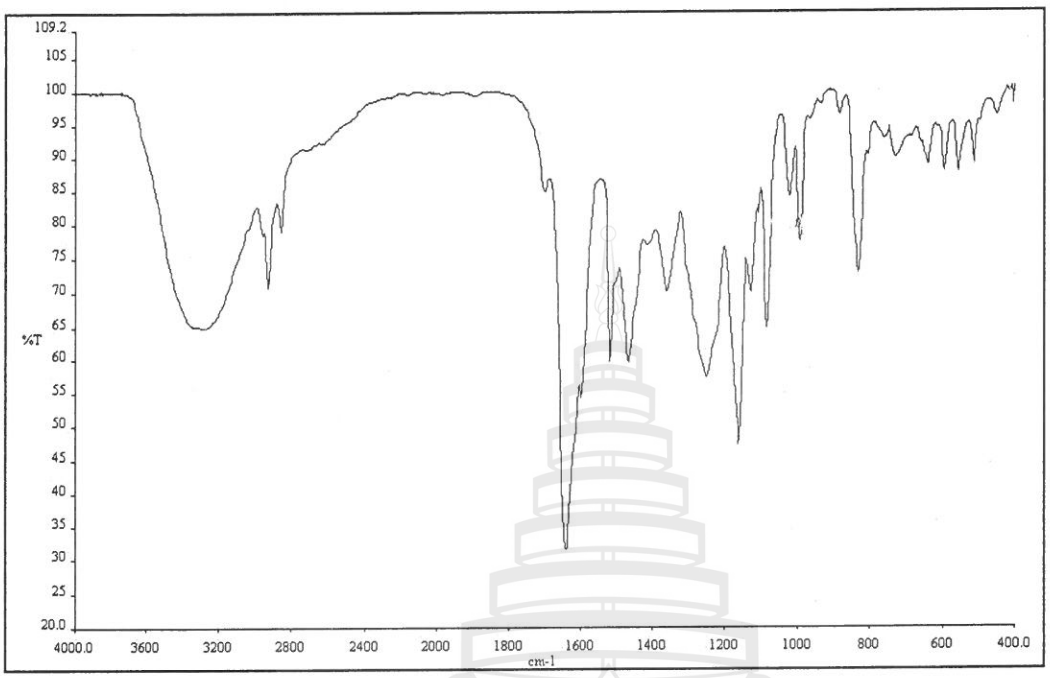
COSY (acetone-d_6) spectrum of **18**



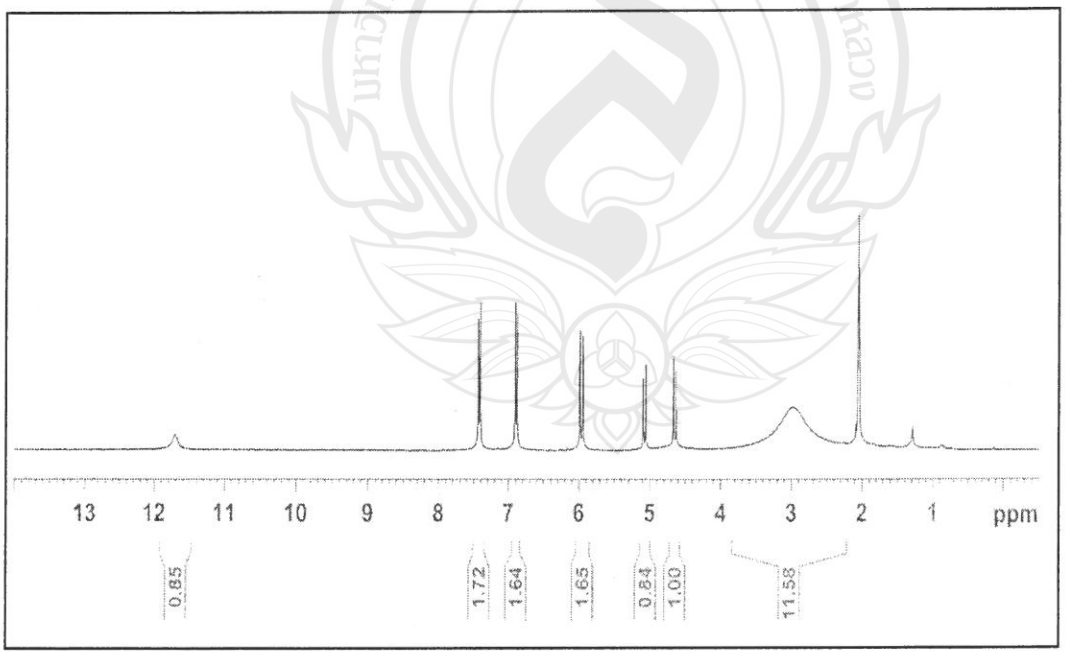
HMBC (acetone-d_6) spectrum of **18**



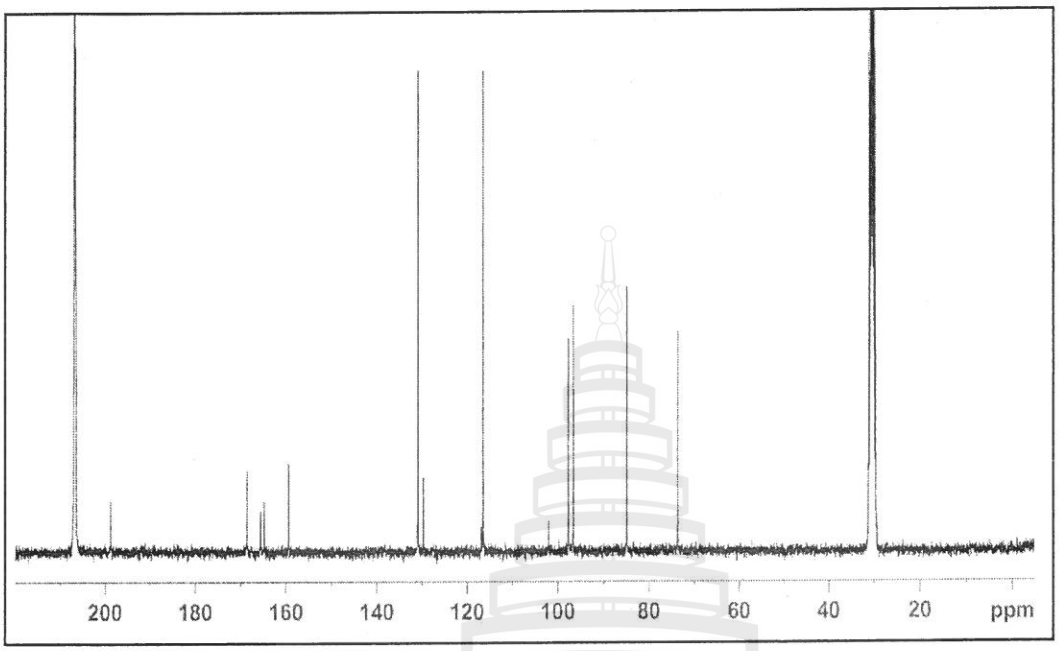
UV (MeOH) spectrum of **19**



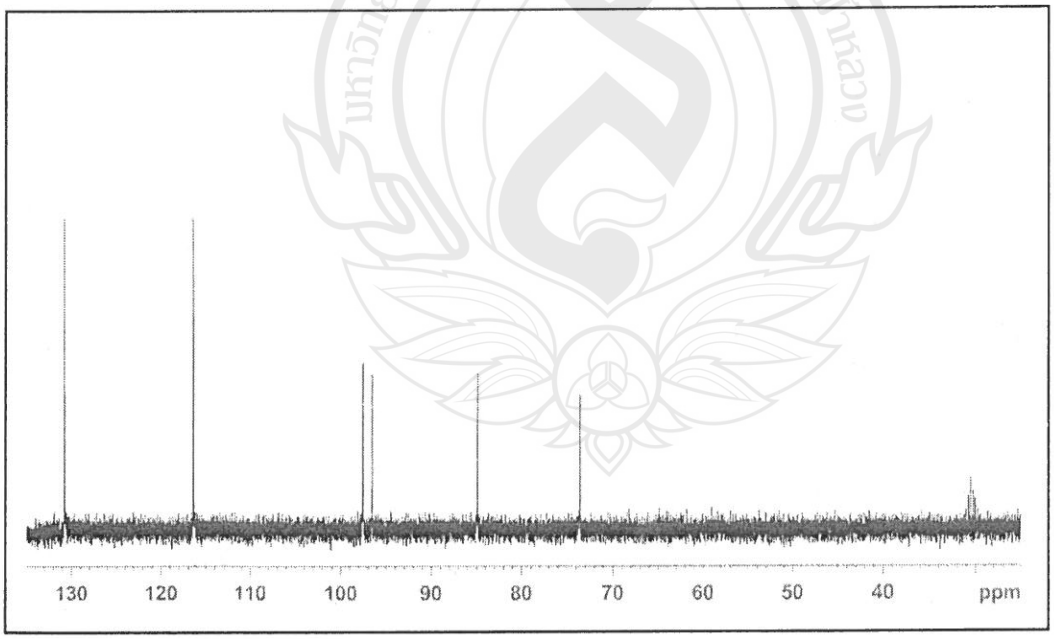
IR (KBr) spectrum of **19**



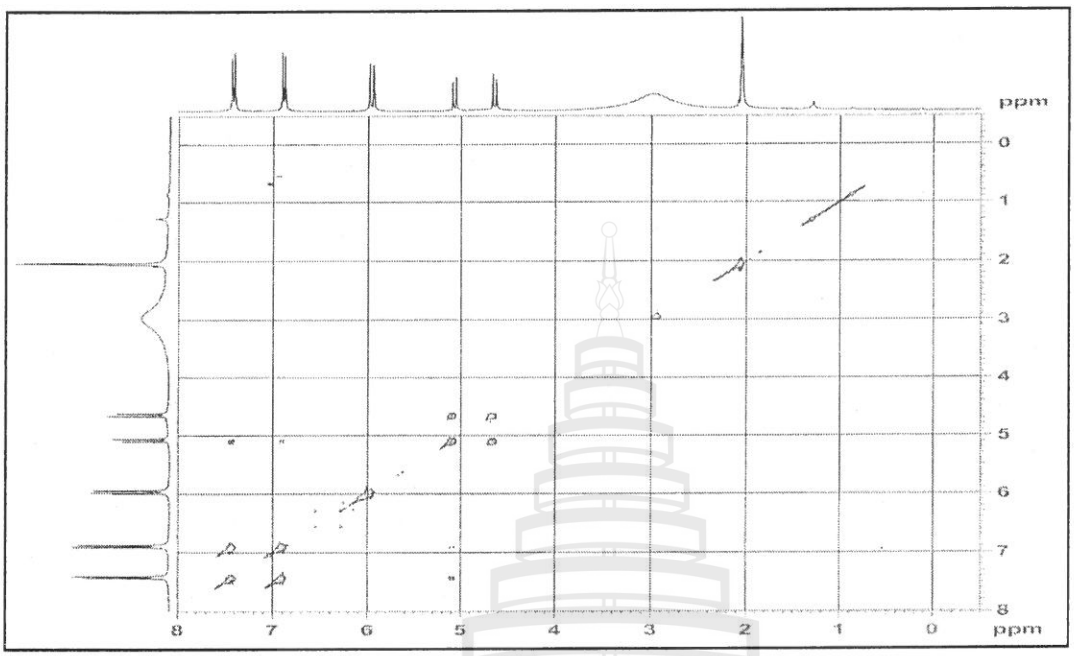
^{1}H NMR (300 MHz, acetone-d_6) spectrum of **19**



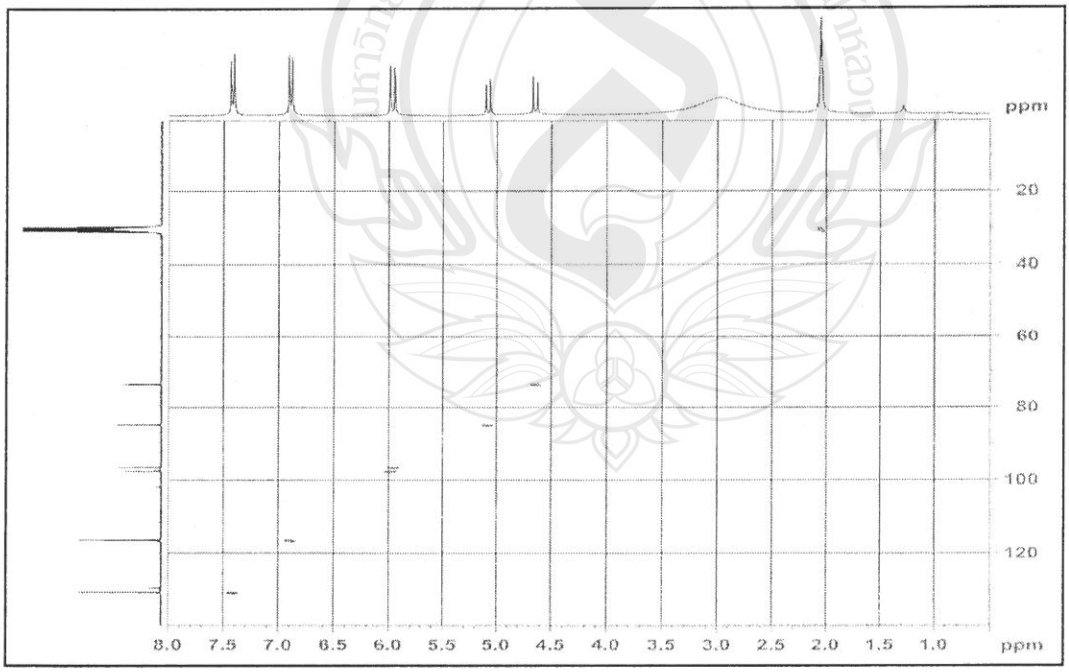
^{13}C NMR (75 MHz, acetone-d_6) spectrum of **19**



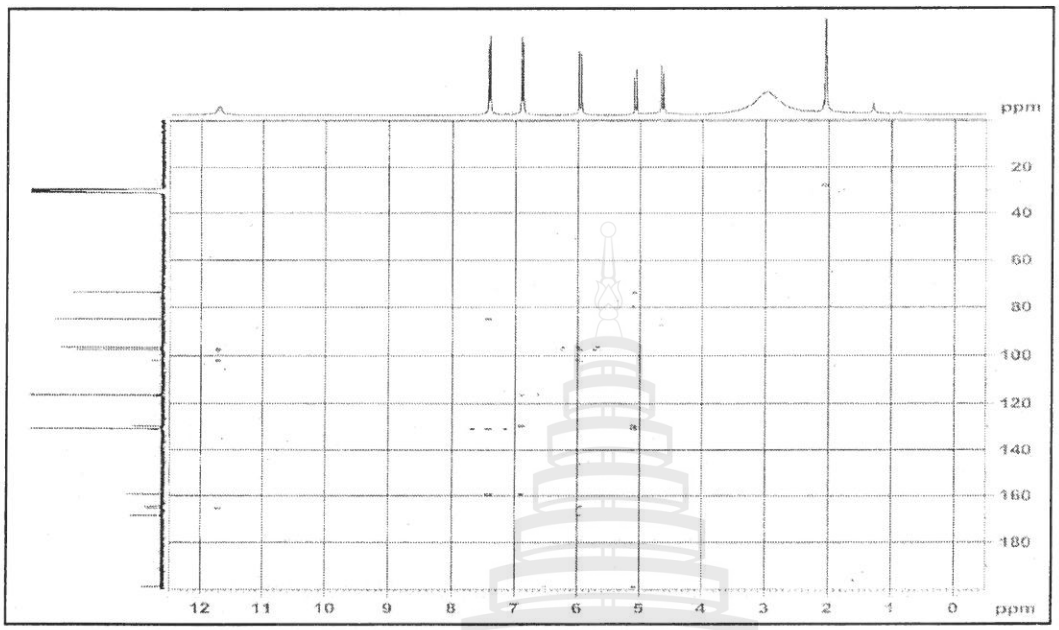
DEPT 90°(acetone-d_6) spectrum of **19**



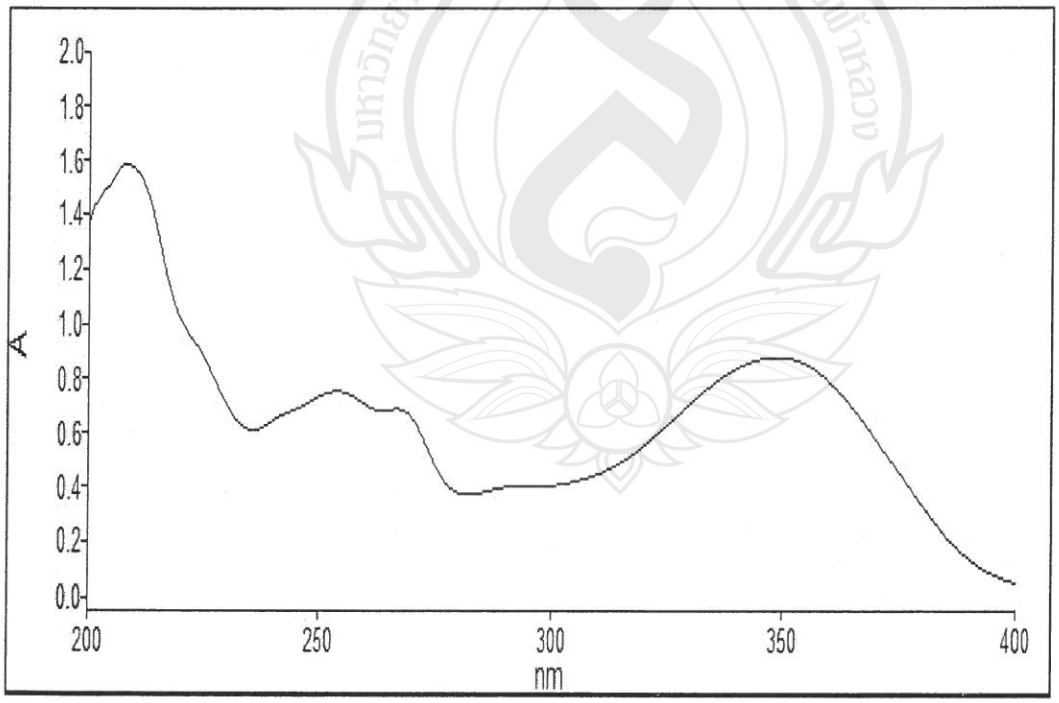
COSY (acetone-d_6) spectrum of **19**



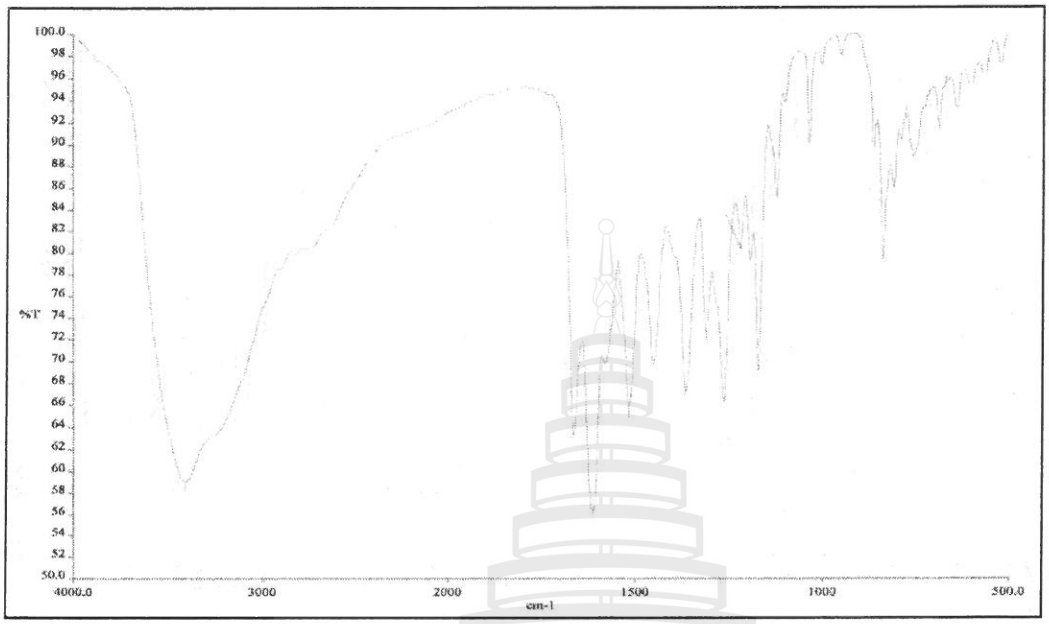
HMQC (acetone-d_6) spectrum of **19**



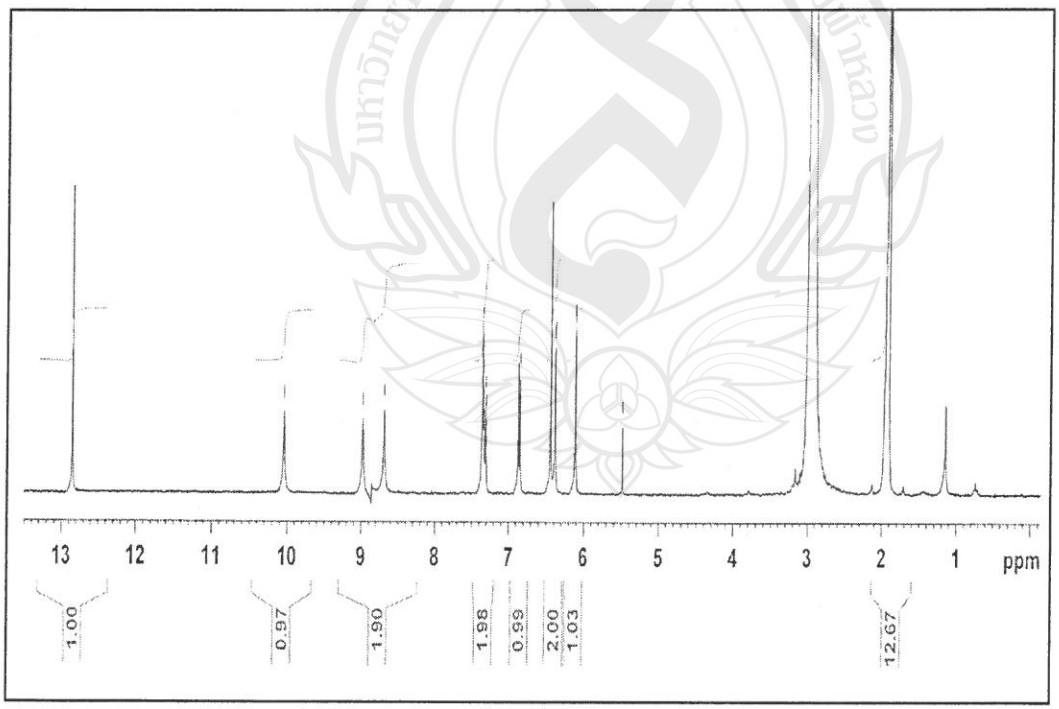
HMBC (acetone-d_6) spectrum of **19**



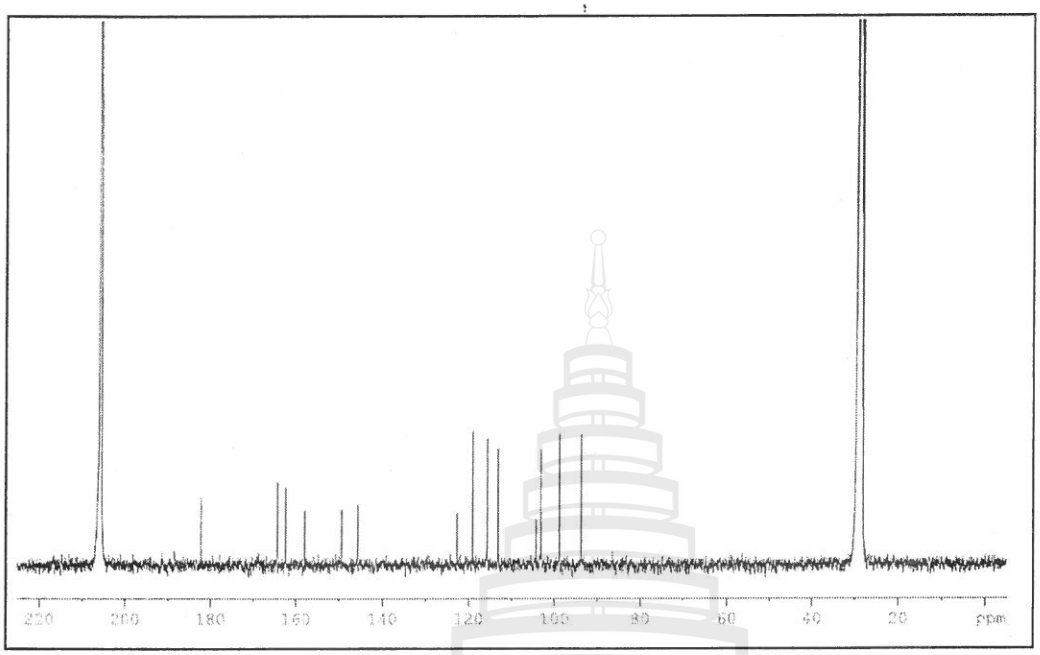
UV (MeOH) spectrum of **20**



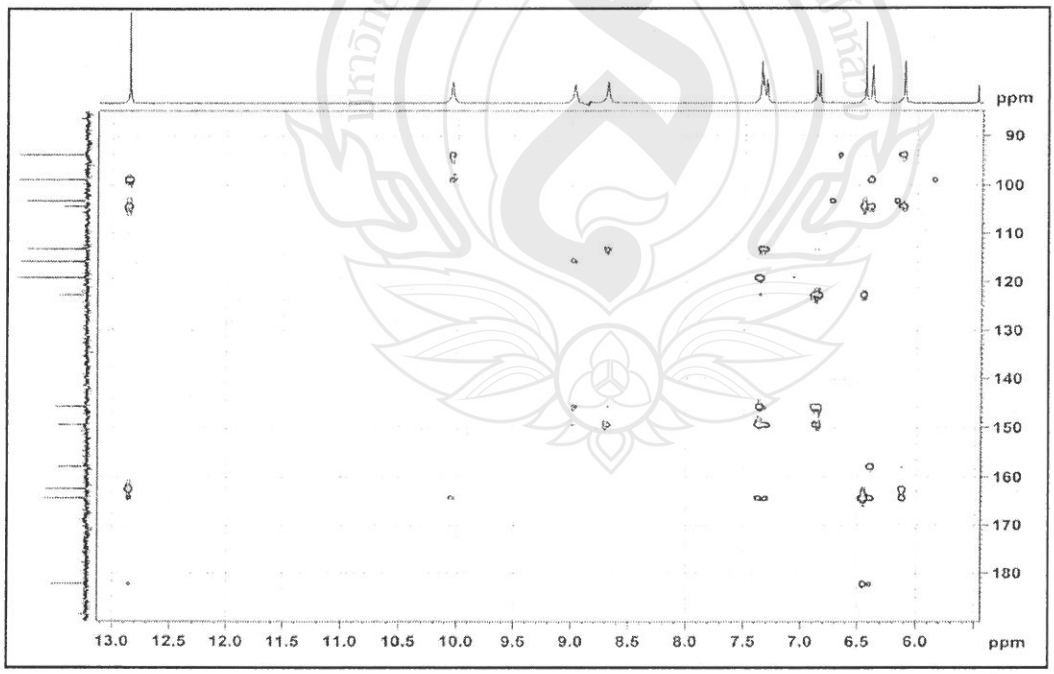
IR (KBr) spectrum of **20**



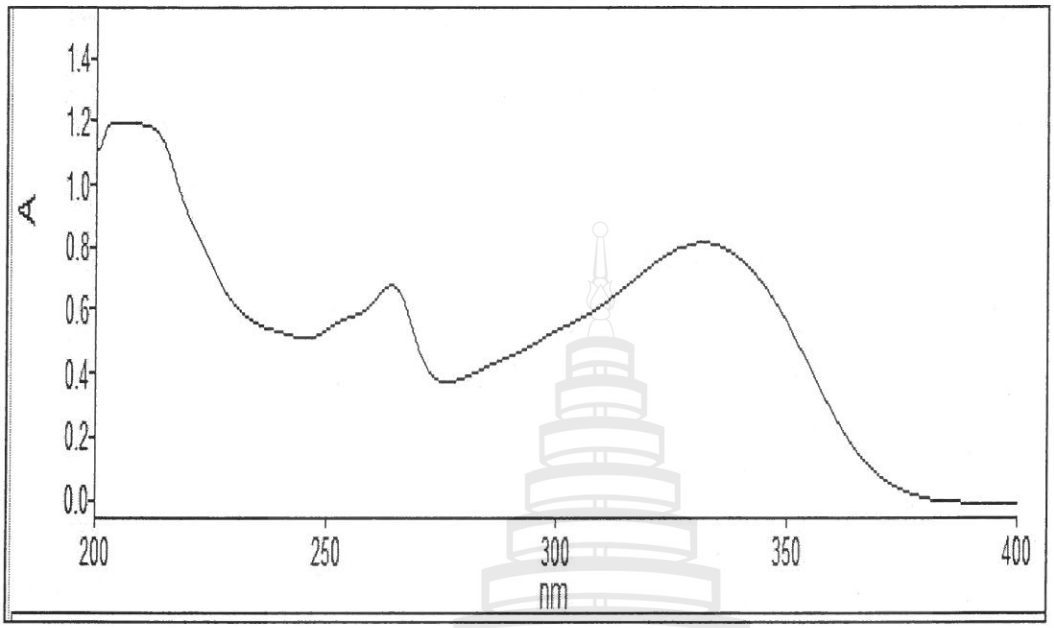
^{1}H NMR (300 MHz, acetone-d_6) spectrum of **20**



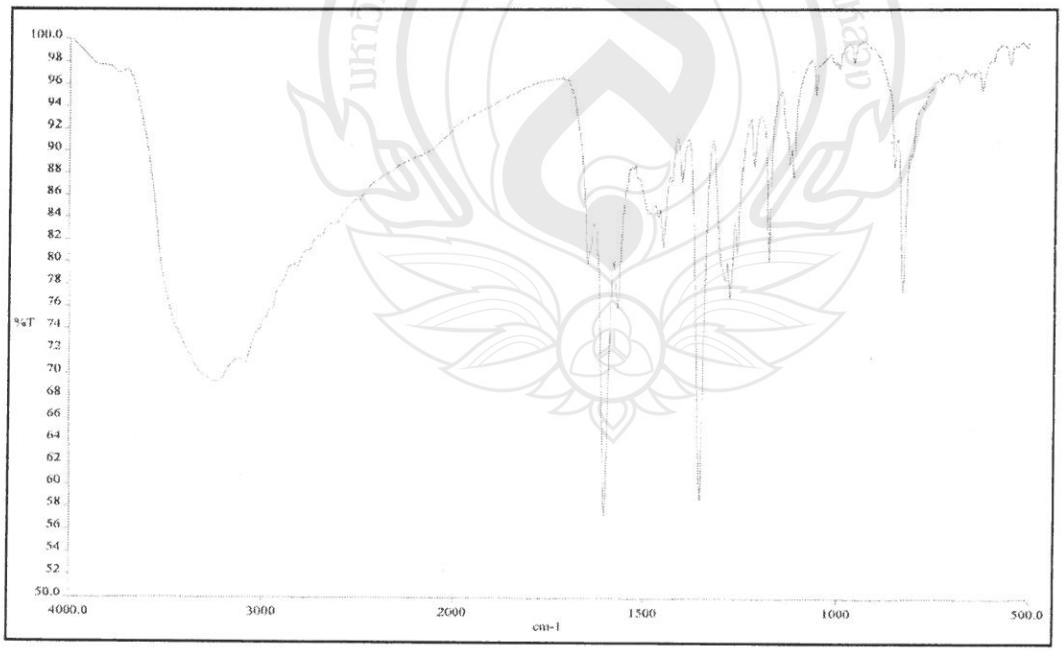
^{13}C NMR (75 MHz, acetone-d_6) spectrum of **20**



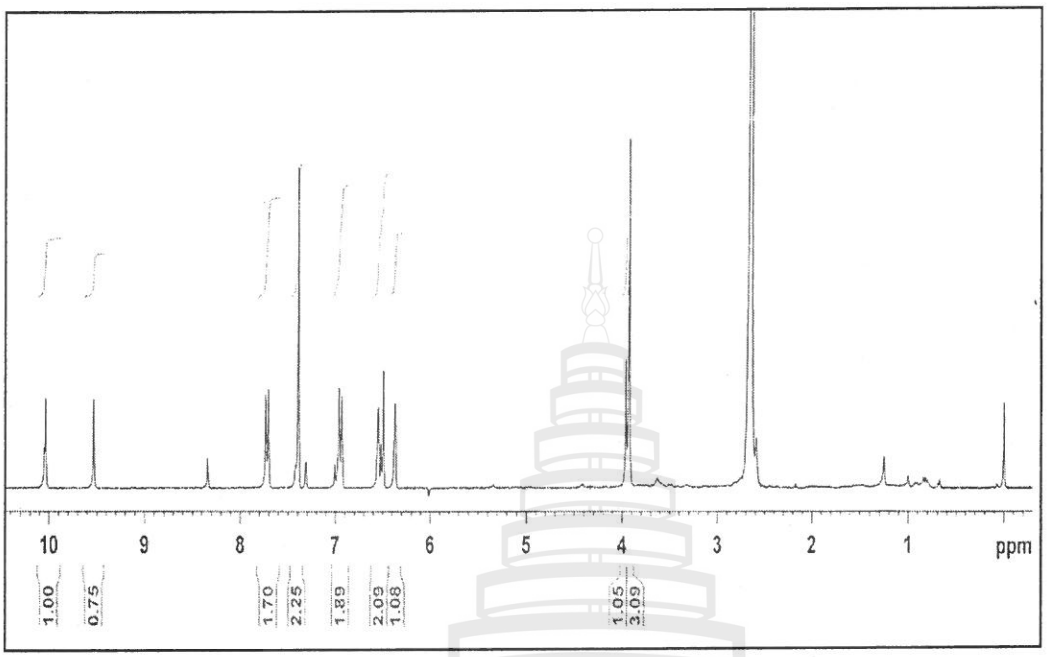
HMBC (acetone-d_6) spectrum of **20**



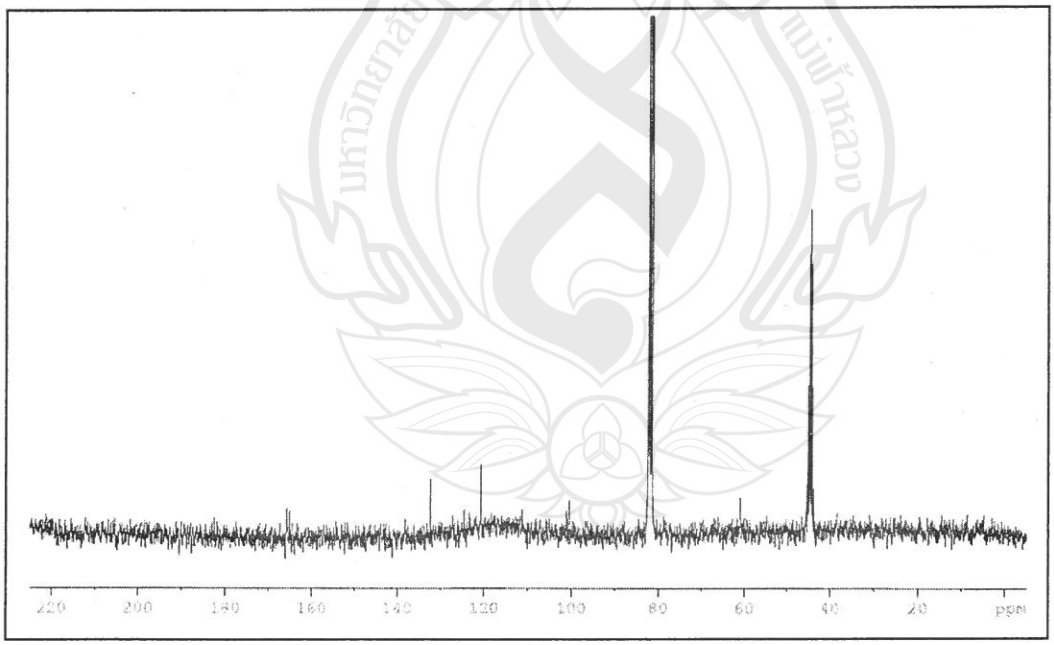
UV (MeOH) spectrum of **22**



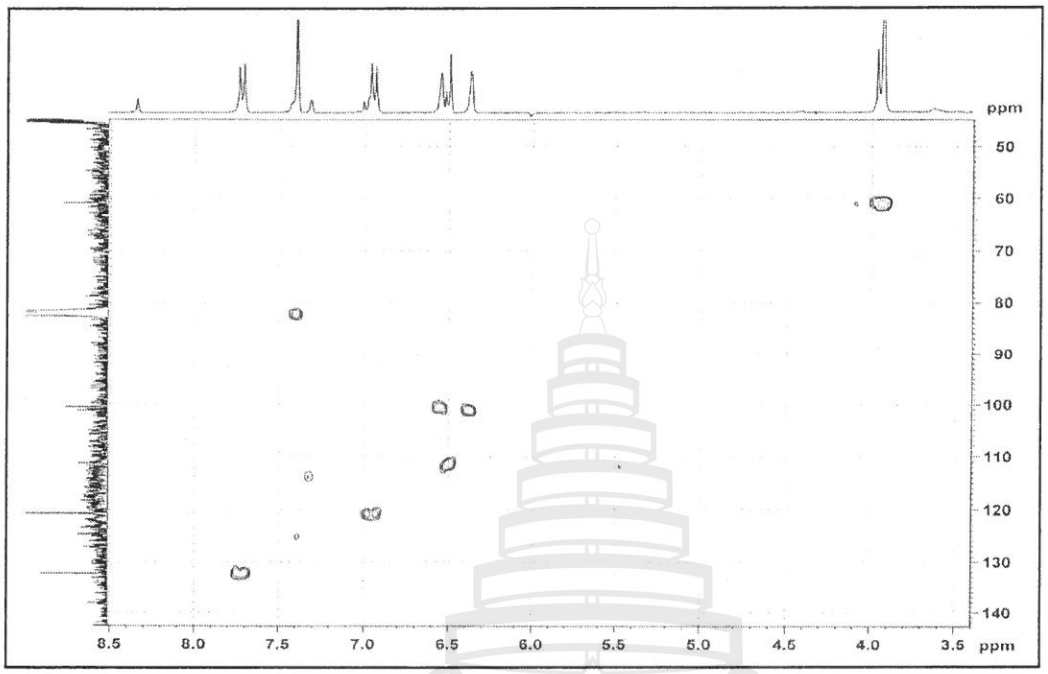
IR (KBr) spectrum of **22**



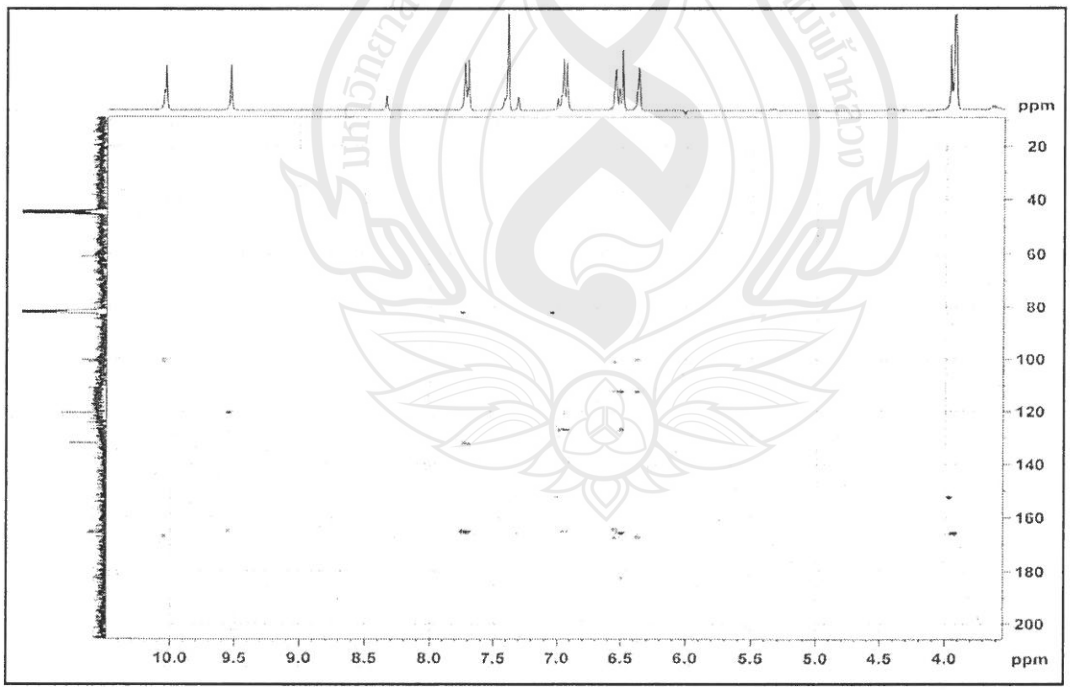
^{1}H NMR (300 MHz, $CDCl_3$+DMSO-d_6)spectrum of **22**



^{13}C NMR (75 MHz, $CDCl_3$+DMSO-d_6)spectrum of **22**



HMQC ($CDCl_3$+DMSO-d_6) spectrum of **22**



HMBC ($CDCl_3$+DMSO-d_6) spectrum of **22**